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PhD Life and Biomolecular Sciences

**IDENTIFICATION OF MOLECULAR
TARGETS OF ONCOGENIC NRAS AND
BRAF INVOLVED IN REGULATION OF
MELANOMA CELL PROLIFERATION**

by

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Biological Science D.

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Doctor in Philosophy in Life and Biomolecular Sciences

3rd April 2007

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ABSTRACT

Mutated BRAF or NRAS genes have been found in ~90% of sporadic melanomas, with a frequency of ~65% of tumors harbouring a mutated BRAF and ~25% of tumors bearing an activated NRAS. However, NRAS^{Q61R} and BRAF^{V600E} are generally mutually exclusive, suggesting that occurrence of both mutations in the same tumor is selected against during tumorigenesis. Analysis of clones isolated from a rare melanoma bearing both mutations showed that the two activating mutations are indeed mutually exclusive at the single cell level. Forced co-expression of the two activating mutations in the same melanoma cell activated senescence and increased susceptibility to cell-mediated cytotoxicity, consistent with a relationship of synthetic lethality between oncogenic NRAS and BRAF. A search for differentially expressed genes in double mutant (i.e. bearing NRAS^{Q61R} and BRAF^{V600E}) vs. single mutated cells (bearing only BRAF^{V600E}) allowed to identify LCN2 and LOXL3, by Microarray analysis, and activated AMPK, by Western blot, as potential targets involved in growth arrest of double mutant cells. AMPK was chosen for further analysis in panels of melanoma cells. Treatment of melanoma cells with either AICAR or Phenformin, two specific activators of AMPK, led to inhibition of growth and of cell cycle progression in different human melanomas that was correlated to the up-regulation of p21 protein. Moreover, cells with activated AMPK showed evidence of induction of senescence. These results suggest that targeting genes associated with oncogenic NRAS and BRAF, and involved in regulation of cell growth and senescence, may allow the development of new therapeutic strategies for advanced melanoma.

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1. INTRODUCTION

1.1 Cancer

1.1.1 Cancer cell alterations.

Cancer is a disease involving dynamic changes in the genome. Several lines of evidence indicate that tumorigenesis in humans is a multistep process where every step reflects genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives. Pathological analyses of a number of organ sites reveal lesions that appear to represent the intermediate steps in a process through which cells evolve progressively from normality through a series of premalignant states into invasive cancers (Foulds, 1954). A large extent of work indicates that the genomes of tumor cells are invariably altered at multiple sites, having suffered disruption through lesions such as point mutations or chromosome rearrangement (Kinzler et al., 1996). Transformation of cultured cells is itself a multistep process, which may vary in different organisms. Taken together, observations of human cancers and animal models argue that tumor development proceeds through a process formally analogous to Darwinian evolution, in which a succession of genetic changes, each conferring one or another type of growth advantage, leads to the progressive conversion of normal human cells into cancer cells (Nowell, 2002).

The wide array of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth (Figure 1.1): self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of

programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Each of these novel capabilities acquired during tumor development represents the successful breaching of an anticancer defense mechanism into cells and tissues (Hanahan et al., 2000). This multiplicity of defenses may explain why cancer is relatively rare during an average human lifetime. The six physiologic changes are shared in common by most types of human tumors.

At genetic level, alterations in three types of genes are responsible for tumorigenesis: oncogenes, tumor-suppressor genes and stability genes. Since mammalian cells have multiple safeguards to protect against the potentially negative effects gene mutations, invasive cancer can develop only when several genes are defective.

Oncogenes are mutated in such a way that renders the gene constitutively active or active under conditions in which the wild type gene is not. Oncogene activations can result from chromosomal translocations, from gene amplifications or from subtle intragenic mutations affecting crucial residues that regulate the activity of the gene product. For example, the most common activating mutation of *BRAF* in human cancers changes a valine to a glutamate at codon 600, a residue within the activation loop of the kinase domain (Davies et al., 2002). An activating somatic mutation in one allele of an oncogene is generally sufficient to confer a selective growth advantage on the cell.

On the other hand, tumor-suppressor genes are targeted in the opposite way by genetic alterations: mutations reduce the activity of the gene product. Such inactivations arise from missense mutations at residues that are essential for its activity, from mutations that result in a truncated protein, from deletions or insertions of various sizes, or from epigenetic silencing. Some recently described tumor-suppressor genes have been

hypothesized to exert a selective advantage on a cell when only one allele is inactivated and the other remains functional: this is the concept of haploinsufficiency (Santarosa et al., 2004). Both oncogene and tumor-suppressor gene mutations operate similarly at the physiologic level: driving the neoplastic process by increasing tumor cell number through the stimulation of cell birth or the inhibition of cell death or cell cycle arrest. The increase can be caused by activating genes that drive the cell cycle, by inhibiting normal apoptotic processes or by facilitating the provision of nutrients through enhanced angiogenesis.

The third type of genes commonly altered in cancer is represented by stability genes or caretakers, which promote tumorigenesis in a completely different way when mutated. This class includes the mismatch repair (MMR), nucleotide-excision repair (NER) and base-excision repair (BER) genes responsible for repairing subtle mistakes made during normal DNA replication or induced by exposure to mutagens. Other stability genes control processes involving large portions of chromosomes, such as those responsible for mitotic recombination and chromosomal segregation (for example, *BRCA1*, *BLM* and *ATM*). Stability genes keep genetic alterations to a minimum, and thus, when they are inactivated, mutations in other genes occur at a higher rate (Friedberg, 2003). As with tumor suppressor genes, both alleles of stability genes generally must be inactivated for a physiologic effect to result.

Mutations in these three classes of genes can occur in the germline, resulting in hereditary predispositions to cancer, or in single somatic cells, resulting in sporadic tumors. The first somatic mutation in an oncogene or tumor suppressor gene that causes a clonal expansion initiates the neoplastic process (Nowell, 2002). Subsequent somatic mutations result in additional steps of clonal expansion and thus in tumor progression (Maley et al., 2004).

Indeed, the best modern definition of a neoplastic cell is a cell that has clonally expanded as a result of somatic mutations (Nowell, 2002; Maley et al., 2004). Germline mutations of these genes can cause cancer predisposition, as a mutation that can contribute to cancer is already present in every cell of these persons. Such individuals therefore often develop multiple tumors that occur at an earlier age than in individuals whose cancer gene mutations have all occurred somatically (Knudson et al., 2002).

In general, cancer gene mutations enhance cell growth. To achieve this effect, several cancer genes directly control transitions from the resting stage G_0 or G_1 to the S replicating phase of the cell cycle. The products of these genes include proteins like CDK4, cyclin D1, RB and p16 (Ortega et al., 2002). The genes encoding RB and p16 are tumor suppressor genes inactivated by mutation, whereas those encoding CDK4 and cyclin D1 are oncogenes activated by mutation. These four genes function in a single pathway in human cancers. Studies on this pathway have shown that the mutations of these genes obey an exclusivity principle, that is, one and only one of the four genes noted above is generally mutated in any single tumor, exactly as predicted if the functional effect of each mutation was similar (Vogelstein, 2000).

The process of tumorigenesis is initiated when a replication competent cell, such as a stem cell or a partially differentiated descendent of a stem cell, acquires a mutation in a 'gatekeeping' pathway that endows it with a selective growth advantage (Vogelstein, 2004). In some cancers, the gatekeeper has been identified (for example *RBI*, *APC* and *NFI* in tumors of the eye, colon and nervous system, respectively). In most common tumors, however, the gatekeeper is not known. It is also not known whether cancers of the lung, breast, prostate, bladder or brain can each be initiated through anyone of several

gatekeeping pathways or through only one. Many of the known gatekeepers were identified through the study of unusual families with predispositions to specific types of cancers (Knudson et al., 2002). As gatekeeping mutations provide fundamental insights into the biology and pathogenesis of particular cancers and are of singular importance to future diagnostic and therapeutic strategies, further research on this topic is actively going on.

In addition to the genes that are mutated in a significant portion of cancers of a given type, there are many other genes that have been implicated in neoplasia but not shown to be mutated. These genes have been shown to be expressed at higher or lower levels than expected in normal cells (Brown et al., 1999; Polyak et al., 2001) and are often associated with epigenetic changes, that is, covalent modifications of DNA or chromatin that are preserved as the cancer cells divide (Jones et al., 2002; Feinberg et al., 2004). Unlike genetic changes, epigenetic changes identical to those found in cancers are often found in normal cells at some stage of development.

1.1.2 Cancer Stem Cells.

Tissues in the body are maintained by somatic stem cells. To maintain homeostasis in the body it is important to keep tight control over stem cell fate. The regulation of the balance between cell renewal and cell death is critical in carcinogenesis process (Sell, 2006). There is increasing evidence that cancer initiation results from accumulative oncogenic mutations in long-lived stem cells or their immediate progenitor, followed by modification of the surrounding microenvironment (Tysnes et al., 2007).

Cancer stem cells, or cancer initiating cells, have been described in the context of acute myeloid leukemia (Lapidot et al., 1994; Bhatia et al., 1997; Bonnet et al., 1997; Blair et al., 1997), breast (Al-Hajj et al., 2003), brain (Singh et al., 2003), bone (Gibbs et al., 2005), lung (Kim et al., 2005), melanoma (Fang et al., 2005) and prostate (Collins et al., 2005). Similarly to normal stem cells, cancer stem cells can self-renew and differentiate into progeny cancer cells. Tumors contain a cellular subcomponent represented by stem cells.

Indirect evidence supports the presence of melanoma stem-like cells (Fang et al., 2005). Melanomas show phenotypic heterogeneity both *in vivo* and *in vitro*, suggesting an origin from a cell with multilineage differentiation abilities; in addition, melanoma cells can differentiate into a wide range of cell lineages, including neural, mesenchymal, and endothelial cells. Like all stem cells, melanoma spheroid cells are also capable of proliferation, differentiation and self-renewal. Moreover, melanoma spheroid cells possess high tumorigenicity capability (Fang et al., 2005).

Such properties suggest a stem cell origin of melanoma. This might have relevant implications for new efficient therapeutic strategies. In fact, the development of cancer therapeutics based on tumor regression may require targeting this important cell population (Wicha et al., 2006). The characterization of a cancer stem cell profile within diverse cancer types may open up new avenues for cancer treatment (Gudjonsson et al., 2005).

1.2 Melanoma.

1.2.1 Melanoma incidence, pathogenesis and etiology.

Worldwide melanoma incidence is sensibly variable depending on different ethnic groups. In particular, incidence rates for cutaneous melanoma have risen faster than those for any other malignancy in Caucasian populations over the last 30 years. Mortality from melanoma is greater than that caused by all other types of skin cancer, especially in men (Giblin et al., 2007). Over these last years, mortality rates have continued to climb, although survival rates are significantly different in different stages of the disease. Human cutaneous melanoma is hardly curable in advanced disease. Tumor thickness and ulceration represent the most powerful predictors of survival. Although surgical excision of thin (<1.0 mm) primary lesions without ulceration (AJCC Stage IA) is associated with a 10-year survival of $86\% \pm 0.9$, survival decreases with increasing thickness of the primary tumor, since this in turn signals for increased probability of lymph node metastases. Ten-year survival in patients with >4 mm primary lesions without ulceration (Stage IIB) is reduced to $\sim 50\%$ (Balch et al., 2001; Balch et al., 2001) and decreases further with development of regional lymph node metastases (Stage III). With the appearance of distant and visceral metastases (Stage IV), 5-year survival drops to less than 10% (Balch et al., 2001). The striking and progressive drop in survival from Stage I to Stage IV highlights the urgent need for achieving a significant advancement on understanding the role of genes expressed/silenced in this disease and that may play a positive or negative role in tumor biology.

Melanoma arises from the malignant transformation of melanocytes, pigment-producing cells found in the basal layer of the epidermis and also in eye (Hurst et al., 2003). Melanocytes derive from neural crest lineage and migrate to the skin during development (Bennett, 1993). Each melanocyte transfers pigment-containing melanosomes through dendritic processes that contact keratinocytes in basal and superficial layers of the skin. In the keratinocytes, the melanin protects the skin by absorbing and scattering harmful solar radiation (Jimbow et al., 1993).

On the basis of histological criteria, melanomas are classified according to their location and stage of progression. Five distinct stages have been proposed in the evolution of melanoma: common acquired and congenital nevi without dysplastic changes; dysplastic nevi with structural and architectural atypia; radial-growth phase (RGP) melanoma; vertical growth-phase (VGP) melanoma; and metastatic melanoma (Figure 1.2). Benign and dysplastic nevi are precursor lesions which progress to in situ melanoma growing laterally and remaining largely confined to the epidermis (RGP). In the next tumorigenesis step, VGP invades the upper layer of the epidermis and beyond, and then penetrates into the underlying dermis and subcutaneous tissue through the basement membrane. The transition from RGP to VGP represents the crucial step in the evolution of melanoma and may be significantly indicative for the clinical outcome of the disease. In fact, the total thickness/height of a primary melanoma lesion is still one of the most predictive parameters for metastatic disease and adverse clinical outcome (Balch et al., 2001).

Melanoma pathogenesis is driven by both genetic and environmental risk factors and its incidence is influenced by skin pigmentation, sun-exposure history and geographical

location, indicating that ultraviolet (UV) light might have a causal role in melanoma development. In fact, upon exposure to UV light, keratinocytes stimulate melanocytes to produce the pigment melanin, which is used to protect the skin from further UV damage (Slominski et al., 2004). Melanoma can be segregated into four groups according to the site of the primary lesion and, therefore, to the amount of UV exposure that is perceived to have induced the cancer (Curtin et al., 2005). Two of these groups arise on sites of high UV exposure, either accumulated owing to chronic exposure throughout life (i.e. chronic sun damage (CSD)), such as when it occurs on the face, or as a consequence of episodes of acute, high-intensity exposure (non-CSD), such as when it occurs on the back or trunk. The other two groups are from sites of low or no UV exposure, such as the palms of the hands or the soles of the feet (acral melanomas) or on internal sites involving mucosal membranes or the uveal tract of the eye (mucosal and uveal melanoma, respectively).

1.2.2 The genetic basis of melanoma.

1.2.2.1 Genetics of melanoma predisposition.

From a genetic point of view, in which neoplastic transformation is viewed as a disease of genes, melanoma progression is associated to identifiable genetic mutations, which drive cancer cells to abnormal growth and dissemination (Chin, 2003). Genetic background may alter both the susceptibility to melanoma development and the disease course. As inherited cancer syndromes have often shed light on the genetic lesions that

govern the genesis of both familial and sporadic forms of a disease, the identification of melanoma susceptibility genes is relevant for improving disease prevention. About 10% of all melanoma cases are familial (Fountain et al., 1990; Rodolfo et al., 2004). Genetic analysis in familial melanoma patients has identified at least two susceptibility genes: CDKN2A and CDK4 (reviewed by Sharpless et al., 2003). Another attractive possible candidate as a melanoma susceptibility gene is melanocortin-1 receptor (MC1R) (Valverde et al., 1996).

The CDKN2A cancer hot spot encodes for two tumor suppressor proteins: INK4A (also known as p16) and ARF (also known as p14). The inhibitor of cyclin-dependent kinase p16 inhibits CDK4/6-mediated phosphorylation and inactivation of RB (Serrano et al., 1993). The alternative reading-frame product of the locus inhibits MDM2-mediated ubiquitylation and subsequent degradation of p53 (Zhang et al., 1998). Therefore, the two products of the CDKN2A locus negatively regulate the RB and p53 pathways, and their loss predisposes to the development of melanoma. The incidence of CDKN2A mutations among melanoma-prone families ranges from 25-40% whereas only 0.2-2% of sporadic melanoma patients harbor germline CDKN2A mutations (Aitken et al., 1999).

Few data are available on p14, the second product of the CDKN2A locus. It has been established that exclusive p14 mutations are very rare and that the gene may be silenced by hypermethylation or by post-transcriptional modifications, but the role of p14 in melanoma progression remains substantially untested. The few published studies suggest that p14 and p16 loss may go together in melanoma progression (Dobrolowsky et al., 2002).

The known CDK4 point mutations are epistatic to INK4A inactivation in melanoma, further supporting the view that the important function of INK4A in melanoma suppression is the regulation of CDK4/6 activity, as the indistinguishable clinical impact of germline CDKN2A (INK4A) and CDK4 mutations testimonies corroborates (Goldstein et al., 2000).

The red-hair color (RHC) phenotype has long been associated with an increased risk for melanoma and it is linked to specific variants of MC1R. The human MC1R is a seven-transmembrane G-protein-coupled receptor (GPCR) for the α -melanocyte-stimulating hormone (α -MSH) expressed on epidermal melanocytes (Garcia-Borron et al., 2005). MC1R is highly polymorphic in human population with variants that differ in signaling strength resulting in the variation of pigmentation phenotypes and skin phototypes (Rees et al., 2003; Sturm et al., 2002). Three common variants of MC1R (R151C, R160W, D294H) are associated with the RHC phenotype (Flanagan et al., 2000) and individuals carrying these alleles have increased sensitivity to UV light (Valverde et al., 1996). At the molecular level, in response to UV light, MC1R is activated by its ligand α -MSH produced by surrounding keratinocytes; the effect is the stimulation of the production of cAMP through the intracellular messenger adenylyl cyclase (AC). The second messenger cAMP activates protein-kinase A (PKA) which translocates to the nucleus where phosphorylates the CREB family of transcription factors. Among these, the microphthalmia-inhibitor transcription factor MITF is activated by the binding through an E box promoter element with PKA allowing for the transcription of pigmentary genes (Busca et al., 2000; Widlund et al., 2003). Several studies have shown MC1R variants

markedly modified the penetrance of mutations at the *CDKN2A* locus (Box et al., 2001) and predispose some individuals to *BRAF* mutations (Landi et al., 2006).

1.2.2.2 Somatic mutations in melanoma.

Neoplastic transformation and tumor progression, in all tissues, is considered as a multi-step process leading to accumulation of genetic and epigenetic changes affecting the function of genes that regulate normal programs of cell proliferation, differentiation and death (Hanahan et al., 2000). Although the genetic basis of sporadic melanoma is not completely understood, recent developments have revealed a remarkably high frequency of mutations affecting *NRAS* and *BRAF* oncogenes in the mitogen-activated protein kinase (MAPK) signaling cascade (Polsky et al., 2003; Pollock et al., 2003).

The MAPK pathway is activated through sequential phosphorylation of a number of kinases to rapidly and reciprocally alter cellular behavior in response to diverse environmental stimuli (Johnson et al., 2002). The extracellular-signal regulated kinases (ERK1 and ERK2) belong to this cascade that is responsible for sensing extracellular stimuli, including UV light (Yanase et al., 2001). Such stimuli activate the RAS family of proto-oncoproteins (HRAS, KRAS, NRAS), which in turn activates the RAF family of serine/threonine kinases (ARAF, BRAF, CRAF), also named MAPK kinase kinase (MAPKKK). Then RAF phosphorylates the MAPK kinase MEK which subsequently activates by phosphorylation the MAPKs ERK1 and ERK2.

Mutated *BRAF* or *NRAS* genes have been found in ~90% of sporadic melanomas, with a frequency of ~65% of tumors harboring a mutated *BRAF* and ~25% of tumors bearing an activated *NRAS* (Davies et al., 2002; Dong et al., 2003). The most common alteration

involving *NRAS* in melanoma is the Q61R transition, resulting from an A182G point mutation, while the most common *BRAF* change is the V600E (T1796A) substitution. *BRAF* and *NRAS* mutations are generally mutually exclusive and, in addition, *BRAF* mutations appear related to progression rather than to tumor initiation, while *NRAS* mutations appear at early stages and remain during progression (Dong et al., 2003; Omholt et al., 2003). In spite of these differences, the very high frequency of alterations and the current knowledge on the key role of the *NRAS*- and *BRAF*-controlled pathways, for several cellular processes, provide compelling evidence as to the involvement of these two oncogenes in the development of sporadic melanoma.

Another causal event in melanoma genesis and progression is represented by the loss of phosphatase and tensin homolog (*PTEN*) tumor suppressor gene. Loss of heterozygosity (LOH) and chromosomal rearrangements in the locus 10q24-26 have been observed in approximately 30-50% of human melanoma cases (Wu et al., 2003). In the 10q regions several tumor suppressor genes are included like *MXI* and *PTEN*. *PTEN* encodes for a lipid/protein phosphatase with dual specificity: by the lipid phosphatase activity *PTEN* signals down the PI3K/AKT pathway regulating G₁ progression and apoptosis, and by the protein phosphatase activity *PTEN* inhibits MAPK signaling (Wu et al., 2003). In melanoma, allelic loss or mutations of *PTEN* have been described in 5-15% of uncultured melanoma specimens and metastases, as well as in 30-40% of established melanoma cell lines (Guldberg et al., 1997; Teng et al., 1997). LOH frequency appears similar in primary and in metastatic lesions, thus suggesting that *PTEN* mutations occur in melanoma initiation, as also suggested by cytogenetic studies. Although a role of

PTEN gene in melanoma pathogenesis is evident in these studies, a better understanding of the *PTEN* signaling events in disease progression need to be obtained.

Recently, *MITF* has been identified as a melanoma oncogene (Garraway et al. 2005). *MITF* amplification has been detected in 10% of primary cutaneous and 15%–20% of metastatic melanomas. *MITF* is a basic helix-loop-helix leucine zipper transcription factor and is considered to be the master regulator of melanocyte biology. Although *MITF* plays a central role in the pigmentation pathway, regulating the expression of melanogenic proteins such as tyrosinase, silver homologue (GP100) and melanoma-associated antigen recognized by T cells-1 (MART-1/Melan-A), it also controls melanoblast survival and melanocyte lineage commitment. *MITF* has been shown to be directly phosphorylated by Erk kinase (Hemesath et al., 1998; Price et al. 1998), triggering its ubiquitylation and subsequent proteasome degradation (Wu et al. 2000; Xu et al., 2000). In addition, mutant *BRAF* has been shown to down-regulate *MITF* levels (Wellbrock et al., 2005), and correspondingly, *MITF* overexpression in a specific context could inhibit growth-stimulatory effects of mutant *BRAF* (Chin et al., 2005). On the other hand, *MITF* is not the only gene implicated in melanocyte survival. Some, such as *Sox10* and *Pax3*, are transcription factors epistatically linked to *MITF* (Garraway et al., 2005). Another group includes growth factor or receptor genes, such as endothelin 3, endothelin receptor B, stem cell factor and c-Kit. The former is a receptor tyrosine kinase, which is activated by mutations in about 2% of metastatic melanomas (Willmore-Payne et al., 2005). The presence of both *BRAF* and c-Kit activating mutations in malignant melanoma suggests that new approaches involving specific tyrosine kinase inhibitors may be relevant to treat this disease.

Other genetic alterations, which commonly are found in melanoma, have the effect to reduce apoptosis. For example, frequent alterations in melanoma are the overexpression of Akt3, of B-cell leukemia/lymphoma 2 (Bcl-2) and of nuclear factor- κ B (NF κ B). In addition, the apoptotic peptidase activating factor-1 (*APAF-1*), which is an essential component of the apoptosome complex, is frequently silenced in advanced melanoma (Soengas et al., 2001).

1.2.3 Function of *NRAS* and *BRAF* oncogenes.

RAS proteins (21 kDa) are guanine nucleotide-binding proteins, which control growth of normal and transformed cells. They cycle between an inactive GDP-bound state and active GTP-bound state, associating with the inner plasma membrane (Figure 1.3) (Boguski et al., 1999). Upon binding of growth factor ligands to receptors at the cell surface, RAS proteins behave as “on/off” switches, coupling extracellular signals to intracellular pathways (Adjei, 2001). In normal cells, the GTP-bound form of RAS (“on” state) is controlled by RAS-specific guanine nucleotide exchange factors (GEFs), while GTPase activating proteins (GAPs) promote hydrolysis of GTP to GDP, switching RAS proteins in the “off” state (McCormick, 1999). Oncogenic activation stabilizes RAS in a GTP-bound constitutively “on” state.

Three *RAS* genes are translated into 4 RAS proteins: HRAS, NRAS, KRAS A and KRAS B. All the RAS proteins have a specific amino acid sequence motif at the carboxyl (C-) terminus, containing a cysteine residue followed by two aliphatic amino acids and either a methionine or serine residue. This motif is essential for the post-translational processing of RAS proteins. A farnesylation process on the C-terminus of RAS protein is needed for

membrane localization and for effective signal transduction capability of the protein (Olsen et al., 2000). Oncogenic mutations of the *RAS* genes are prevalent in a large array of human cancers, including pancreatic adenocarcinoma ($\approx 80\%$), colorectal adenocarcinoma ($\approx 35\%$), lung cancer ($\approx 20\%$), follicular or undifferentiated carcinomas of the thyroid ($\approx 50\%$) and various hematological malignancies (Bos, 1989; Reuter et al., 2000). The most commonly observed mutations occur in hot-spot critical for RAS regulation, which are codons 12, 13 and 61 (Sprang et al., 1997). The effect of the mutations is to prevent normal cycling activity of RAS by decreasing the ability of the protein to complex with GAP. Since RAS-GTP remains intact, the growth-stimulatory signal is prolonged even in the absence of growth factor receptor-ligand binding. RAS can also be activated in tumors by loss of GAPs, as in the case of neurofibromin (NF1) whose loss results in RAS activation (Weiss et al., 1999) or through the over-expression of growth-factor-receptor tyrosine kinases, as the case of EGFR and ERBB2 which are frequently overexpressed in many types of cancer (Mendelsohn et al., 2000).

A multitude of signaling pathways have been linked to RAS activation, among which, the best characterized are the mitogen-activated protein (MAP) and the phosphoinositide 3 (PI3) kinases pathways (Figure 1.4).

BRAF acts downstream of RAS proteins in the RAS-RAF-MEK-ERK MAP kinase (MAPK) pathway. BRAF encodes a serine/threonine kinase acting in the MAPK pathway to transduce signals from RAS, through MEK (Mercer et al., 2003). In mammals, there are three highly conserved RAF genes, ARAF, BRAF and CRAF (Pritchard et al., 1995; Hagemann et al., 1999). BRAF exists in a number of splice variants and produces proteins that range from 70 to 100 kDa (Barnier et al., 1995). The three genes share a

common architecture of three conserved regions, CR1 and CR2 in the N-terminus and CR3 in the C- terminus (Figure 1.5). Within CR1 there is the RAS binding domain (RBD) and a cystein rich-domain (CRD). Phosphorylation of RAF proteins plays an important role in their regulation, with phosphorylation sites being located both in the regulatory domain sited in the N-terminus and the kinase domain housed in the CR3 domain. In the CR3 domain another motif important for RAF activity is present and is called N-region or negative-charge regulatory-region (Figure 1.5). Unlike in ARAF and CRAF, where this site needs to be phosphorylated for full kinase activity, in BRAF it is constitutively phosphorylated thus constitutively “charged”. As a consequence, BRAF is fully activated by RAS alone whereas ARAF and CRAF require both RAS and SRC for full activation (Marais et al., 1997; King et al., 2001). The fact that BRAF requires fewer steps to become activated can explain why it is the isoform that is primarily responsible for signaling between RAS and MAPK in the majority of cells and also why it is more susceptible to mutations in cancer. Recently, it has been shown that both wild type and mutant BRAF can activate CRAF, following a direct binding of BRAF to CRAF and a subsequent trans-phosphorylation of CRAF by BRAF (Wan et al., 2004; Garnett et al., 2005). Wild type BRAF binds to CRAF in a RAS-dependent manner, whereas mutant BRAF binds in a RAS-independent manner (Garnett et al., 2005; Weber et al., 2001). Conversely, CRAF can not activate BRAF (Garnett et al., 2005), but both BRAF and CRAF subsequently signal to MEK.

The majority of the mutations that have been found in BRAF are somatic changes that are presumed to be induced by environmental factors (Davies et al., 2002). The most common BRAF mutation, which is found in more than 90% of cases of cancer involving

this gene, is the glutamate versus valine substitution at codon 600 (V600E) (Davies et al., 2002). Upon its oncogenic activation, BRAF induces constitutive ERK signaling through hyperactivation of the RAS-MEK-ERK pathway and constitutive Nuclear factor kappa-B (NFkB) signaling, thus stimulating proliferation, survival and transformation. Although it can stimulate many of the hallmarks of cancer, thus being identified as an oncogene (Garnett et al., 2004), BRAF is yet mutated in up to 80% of benign nevi (Pollock et al., 2003; Dong et al., 2003). Indeed, BRAF^{V600E} induces senescence in melanocytes, through transcriptional upregulation of the cell cycle inhibitor p16 (Michaloglou et al., 2005; Gray-Schopfer et al., 2006). Thus, BRAF plays an important role in cancer induction, maintenance and progression; however, by itself oncogenic BRAF is not sufficient for cancer and must cooperate with other processes to induce the fully cancerous state.

1.2.4 Consequences of NRAS and BRAF activation on gene expression.

The presence of one or the other of these activated oncogenes in neoplastic cells has remarkable effects on expression of several genes, since the permanent “on” state of the two oncogenes impacts on expression of transcription factors controlled not only by the MAPK pathway (common to both NRAS and BRAF), but even by the Rac/Rho and the PI3K cascades (under the influence of NRAS) (Khosravi-Far et al., 1998). In particular, activation of the MAPK pathway influences the activity of several transcription factors, among which the most important is Fos, which in turn can regulate expression of a number of genes associated with cell proliferation, differentiation and survival, cell cycle regulation and integrin signaling. Moreover, recently, it has been shown that a component of the AP-1 transcription factor is one of the targets of RAS oncogene in fibroblasts

(Casalino et al., 2003). The relevance of the AP-1 pathway, for tumor biology, is underscored by its role in regulating expression of genes coding for extracellular matrix components, proteolytic enzymes and their inhibitors (Poser et al., 2004; Meadows et al., 2004; Castelli et al., 1994). Two additional G-proteins, Rac and Rho, are implicated in the regulation of the active cytoskeleton and have been shown to be activated by RAS (Hanahan et al., 2000). These proteins control critical cellular processes such as the formation of focal adhesion points, filopodia, stress fibers and membrane ruffling, thus indicating a crucial role for processes as cell migration and invasion (Adjei, 2001). One additional RAS effector is PI3K, which on one hand activates Rac and on the other hand acts on Akt, which has numerous downstream survival and death factors targets (Adjei, 2001). Interestingly, it has been recently demonstrated that RAS, possibly through the PI3K pathway, can activate angiogenic phenotype in primary endothelial cells and can control cell migration and metastasis (Meadows et al., 2004). Moreover, there are several other RAS effectors, including PKC ζ , PLC and RalGDS, whose role in RAS signaling remains yet to be understood (Adjei, 2001). In addition, there are distinct lines of evidence suggesting that several transcriptional targets of oncogenic NRAS and BRAF may play a direct or indirect role in regulation of anti-tumor immunity and in the development of tumor escape mechanisms. For example, mutant NRAS has been shown to increase transcription of genes such as apoptosis inhibitors, melanoma-associated antigens and cytokines like IL-1, IL-6 and TNF- α . Moreover, constitutively activated MAPK, resulting from oncogenic NRAS or BRAF, promotes suppression of JAK-STAT transcriptional activity (Krasilnikov et al., 2003). STATs transduce signals from a large array of cytokines (Kisseleva et al., 2002) and several of these factors are produced by

melanoma cells or by the normal surrounding tissues. As a result of STAT activation, transcription of several genes is promoted. Interestingly, STAT regulated genes code not only for proteins that contribute to regulation of proliferation and apoptosis, but even to co-stimulation of T cell mediated response, as exemplified by the recent identification of STAT-1-dependent induction of CD40 in endothelial cells. Thus, it is also conceivable that presence/absence of either an activated NRAS or an activated BRAF in melanoma cell may impact on the expression of genes that are not only related to the malignant phenotype but could even play a role in development and regulation of immunity at tumor site.

A wealth amount of sequence information, with few overlap among the different sets of data, is available on transcriptional changes related to the constitutive activity of mutant RAS genes in rat fibroblasts or to the inducible activity of RAF in human epithelial cells (Zuber et al., 2000; Schulze et al., 2001). It is however known that the cellular context and the type of RAS isoform are important determinants in RAS signaling. Indeed different outputs of RAS oncogenic isoforms as well as distinct effector type recruitment were documented in different cell types.

1.2.5 Gene profiling in human melanoma.

Several studies have investigated whether human melanoma can be classified into distinct subsets on the basis of their gene profile resulting from microarray analysis. By such approaches, evidence for profiles associated with tumor stage and/or biological behavior has indeed been obtained (Polsky et al., 2003; Carr et al., 2003). To this end, microarray expression profiling has been used to examine the sets of molecular changes

that occur in melanoma. Microarrays were used to distinguish different stages of melanoma. A comparison of naevi, primary melanomas and metastatic melanoma samples revealed that these lesions can be distinguished one from each other (Haqq et al., 2005). A molecular signature of invasion in cutaneous melanoma has been identified (Bittner et al., 2000).

On the other hand, less information is available regarding the consequences, for gene expression, of the presence of NRAS or BRAF mutations in the neoplastic cells. Thus, more comprehensive analysis is required to better understand the contribution made by BRAF and NRAS mutations to melanoma progression, prognosis and response to therapy. However, several studies have focused on defining gene profiles regulated by oncogenic BRAF and NRAS. In a recent study that considered the expression profile of a number of melanoma lines whose NRAS or BRAF mutation status was known, presence of the V600E activated BRAF oncogene was linked to a specific gene expression signature made of as few as 83 genes (Pavey et al., 2002). In one study (Bloethner et al., 2005) of 10 melanoma cell lines, 65 genes were shown to distinguish wild type *BRAF* from *BRAF*^{V600E} lines, whereas expression of 109 genes changed when wild type *NRAS* and *NRAS*^{Q61R} lines were compared. Fifty-six genes were common to both the *BRAF* and *NRAS* sets, and many of these genes encode members or regulators of the RAS–RAF–MEK–ERK pathway, whereas others are involved in metastasis or invasion. Despite the fact that it was possible to distinguish oncogenic *NRAS* melanomas from wild type *NRAS* melanomas, it was not possible to distinguish *BRAF* and *NRAS* melanomas from each other.

Microarrays can clearly be used to distinguish different forms of melanoma, but to date this approach has not convincingly allowed for identifying a BRAF or NRAS signature. However, these studies suggested that the transcriptional consequences resulting from mutation of BRAF or NRAS are different, presumably for their differential capacity to transduce signals through the various effectors. Indeed, more studies are therefore required to allow us to harness the power of this approach.

1.2.6 Melanoma resistance to apoptosis.

Despite efforts to promote sun protection behaviors, melanoma incidence continues to increase. The prognosis of advanced melanoma remains extremely poor in spite of treatment advances, emphasizing the importance of exploring additional preventive measures. One of the major consequences of the constitutive activation of the MAPK and PI3K/AKT signal transduction pathway in tumor cells is the induction of tumor cell resistance to apoptosis. Indeed, human melanoma has been showed to be chemoresistant *in vivo* to a wide range of therapeutics acting with different mechanisms (Soengas et al., 2002). These mechanisms often depend on the acquisition of defects in the cell death pathways. Several studies over the past 10-15 years shed light into the mechanisms of melanoma resistance to apoptosis promoted by several chemotherapeutic drugs (Okada et al., 2004). Thus, to target and reverse this antiapoptotic mechanism is attractive as a component of multiple therapies.

Apoptosis is mediated by a sequence of events that result in cell death. In cancer cells there is often an imbalance in favor of antiapoptotic signals, which confers cells a resistance to chemotherapeutic drugs and radiation. Bcl2, a family of proteins, plays an

important role in cancer cell survival (Kim et al., 2005). Overexpression of Bcl2 has been observed in many types of solid neoplasms and hematologic malignancies, including melanoma. Nearly 90% of melanomas present overexpression of this anti-apoptotic protein (Selzer et al., 1996). Members of the Bcl2 gene family as well as the inhibitor of apoptosis protein (IAP) families have been successfully targeted to render tumor cells more susceptible to apoptosis (Klasa et al., 2002; Jansen et al., 1998). To this end, Bcl2 protein expression can be directly down-regulated by antisense oligonucleotides (Kirkwood et al., 2005). An alternative approach to overcome tumor cell resistance to apoptosis is to inhibit the function of IAPs. One of these proteins is survivin, which has been found to be highly expressed in most cancers. In preclinical models small molecules have been demonstrated to suppress the function of survivin, and that has now entered a Phase II trial in several solid tumors including melanoma (Becker et al., 2006).

1.2.7 Targeted therapies for melanoma treatment.

A recent approach for cancer therapy is represented by targeted drugs. In the last decade researchers have focused on the explanation of an altered signal network in cancer cell. The identification of pathways involved in growth regulation, which are important for cancer biology, allowed to act with specificity at molecular level. Protein kinases transmit signals to the nucleus through intracellular cascade. The activity of kinases involved in transmission of proliferation signals in cancer cells often becomes more intense. Kinase inhibitors are generally small molecules designed to act with specificity on intracellular protein-kinases. In particular two oral drugs, sorafenib and imatinib

mesylate, and an intravenous drug, temsirolimus, were tested in melanoma patients. MAPK pathways have been found to be altered in various malignancies (Davies et al., 2002). Given its high rate of mutation in melanoma cells (Sathyamoorthy et al., 2003), *BRAF*^{V600E} is a suitable target for antitumor therapy (Brose et al., 2002). Sorafenib (BAY 43-9006), a bi-aryl urea, is a small molecule originally synthesized for BRAF and CRAF inhibition. It targets the ATP-binding site of the kinase and, at low concentrations, it inhibits *in vitro* both wild type and mutant BRAF. Moreover, sorafenib also inhibits other tyrosine-kinase receptors involved in angiogenesis and tumor progression, including vascular endothelial growth-factor receptor (VEGFR)-2, VEGFR-3, c-Kit and platelet-derived growth-factor β (PDGFR- β).

Other drugs targeting MAPK pathway are the Ras farnesyl transferase inhibitors, specifically designed to block an essential post-translational modification of RAS (Downward, 2003).

Since beside the RAS-MAPK pathway also the PI3K-Akt pathway contributes to melanoma cell survival, PI3K signaling is also a good target for melanoma treatment. Chemical agents, which target PI3K, Akt, mTOR and other downstream components of this pathway, are being developed (Dancey, 2006).

1.3 Oncogene-Induced Proliferative Arrest.

Normal balance between cellular proliferation and cell death is tightly regulated in order to maintain structural and functional stability of the tissues of multicellular organisms. Indeed, a complex machinery of antiproliferative signaling allows proliferation only under particular circumstances to avoid the undesirable effects of excessive cellular proliferation. To this end, specific cell cycle checkpoints preserve genomic integrity and cell survival. Cellular differentiation and apoptotic cell death are similarly subject to strict regulation. Upon the disruption of the tight regulation of cell cycle, cells proliferate without control and may enter and progress on the road to cancer. The oncogenic mutations leading to cancer development generally result in enhanced growth-promoting signals, disrupted anti-proliferative signals, or defective pro-apoptotic signaling.

Molecular mechanisms leading to the cessation of growth of premalignant or benign neoplasms play relevant role in the prevention of cancer. *In vitro* studies revealed that, under certain circumstances, oncogenic signaling can elicit, paradoxically, a potent growth arrest response (Serrano et al., 1997). The irreversible arrested growth is characterized by distinct changes in the cellular phenotype and the phenomenon is generally called senescence. Recent data supported the notion that oncogene-induced senescence might be an *in vivo* mechanism that contributes to protection against cancer (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Michaloglou et al., 2005). Moreover, senescence has emerged recently as a drug-responsive program able to influence the outcome of cancer chemotherapy. Since the drug amounts required for induction of proliferation arrest are much lower than those necessitated for induction of

apoptotic cell death, forcing cancer cells to undergo senescence may represent a less aggressive approach to control tumor progression. However, simultaneously the ability of cancer cells to escape senescence and become drug resistant must be inhibited. Therefore, a clear understanding of the mechanisms that govern drug-induced senescence is critical and can lead to discovery of novel approaches to suppress drug resistance (Rebbaa, 2005).

1.3.1 Replicative and premature senescence.

Normal somatic cells have a limited life span and cannot proliferate indefinitely in culture (Shay et al., 2000). After a certain number of cell divisions, non transformed cells *in vitro* spontaneously stop proliferating. This phenomenon is called the Hayflick limit (Hayflick, 1965). Cultured cells that reach the Hayflick limit become large and flat, are often vacuolated, and commonly activate senescence-associated acidic β -galactosidase, which has recently been suggested to correspond to lysosomal β -D-galactosidase (Lee et al., 2006; Dimri et al., 1995). Senescence-associated acidic β -galactosidase activity can be visualized by a cytochemical reaction resulting in blue precipitate which is a widely used marker of cellular senescence and other types of cellular stress (Dimri et al., 1995). An additional characteristic of senescent cells is represented by the senescence-associated heterochromatin foci (SAHF), which are stretches of transcriptionally silenced DNA associated with a specific modification of histones (Narita et al., 2003). The Hayflick limit results from the fact that at each round of DNA replication, telomeres become slightly shorter and are eventually dysfunctional (Shay et al., 2000). When they reach a critical minimal length, a DNA damage response is triggered. This response is associated

with chromosomal instability, senescence and loss of cell viability. A causal role for telomeres in senescence became evident from the discovery of telomerase, a ribonucleoprotein that elongates telomeres, thereby blocking the progressive shortening of the telomeres of proliferating cells (Harley et al., 1990). Telomerase activity allows various cell types to proliferate beyond the usual maximum number of cellular divisions. Telomere shortening might constitute a generic way to avoid the growth of cancer, since it emerges whenever a cell has used up a maximum number of allowed divisions. Instead, in cancer cells often there is an inappropriate activation of telomerase (Crute et al., 1998) or an alternative mechanism that similarly results in telomere lengthening (Muntoni et al., 2005). Whereas short telomeres can suppress tumorigenesis in mice (Gonzales-Suarez et al., 2000), forced expression of telomerase contributes to malignant transformation of primary cells *in vitro* in the presence of cooperating oncogenes (Hahn et al., 1999). Indeed, there is a strong link between the loss of telomere-associated senescence and cancer. In contrast to somatic cells, most cancer cells have extended or infinite life span. Escape from senescence during neoplastic transformation has been linked to inactivation of the tumor suppressor p53 and to constitutive activation of telomerase (Duncan et al., 1997; Stampfer et al., 2003). Although cancer cells are less sensitive to senescence induced by physiological conditions, they undergo to irreversible growth arrest when subjected by various cellular stresses, including DNA damage, by most artificial culture conditions and by uncontrolled oncogene activation. Indeed, a number of studies have demonstrated that exogenous stresses such as UV and IR irradiation, H₂O₂, hypoxia, and various chemicals, induce a senescence phenotype that is mainly characterized by enhanced expression of the cell cycle inhibitors p16, p21, p27 and disruption of

lysosomal function through enhanced activity of the senescence associated β -galactosidase (SA- β -Gal).

Approximately 20 years ago, it was noted that in untransformed fibroblasts, an activated mutant of the *RAS* gene induced the arrest of cell growth rather than oncogenic transformation (Franza et al., 1986). However, if the cells already harbored certain oncogenic mutation, the same mutant *RAS* gene contributed to oncogenic transformation (Land et al., 1993). Only after about 10 years, in 1997, Serrano and colleagues noticed that the type of proliferative arrest elicited in young diploid fibroblasts by mutant *RAS* exhibited many features of replicative (telomere-associated) cellular senescence, including stable maintenance of cell cycle arrest (Serrano et al., 1997). Thus, senescence could be induced in non malignant cells before telomeric shortening. Such phenomenon was called premature senescence. Premature, or oncogene-induced, senescence is an irreversible process.

The findings about *in vitro* senescence quickly led to speculation about possible *in vivo* correlations that might provide protection against cancer and account for the phenomenon of proliferative arrest in benign tumors (Bennett, 2003; Mooi et al., 2002; Campisi, 2001; Bringold et al., 2000; Lowe et al., 2003). Nevertheless, it should be noticed that many differences exist between *in vitro* and *in vivo* cell conditions. For example, the different microenvironment of cultured cells in respect to the *in vivo* microenvironment (Sherr et al., 2000) can cause a phenomenon called culture stress, which is a distinct process from senescence phenomenon. However, recently several studies have provided substantial support for the notion that oncogene-induced senescence is a physiologic mechanism for

protection against cancer (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Michaloglou et al., 2005; Lazzerini et al., 2005).

Oncogene-induced senescence is accompanied by the activation of a tumor suppressor network. It has been shown that cell senescence can be triggered *in vivo* not only by oncogene activation, but also by inactivation of tumor suppressors such as *PTEN* (Chen et al., 2005). Indeed, in cultured cells, premature senescence is caused by the activation of a set of tumor suppressor genes that are often inactivated in human cancer and various cancer-susceptibility syndromes: *INK4A* (inhibitor of cyclin-dependent kinase 4) (Gruis et al., 1995; Kamb et al., 1994), *ARF* (alternative reading frame) (Randerson-Moor et al., 2001; Rizos et al., 2001), *p53*, and *RB* (the retinoblastoma tumor suppressor gene) (Palmero et al., 1998; Tanaka et al., 1994; Peeper et al., 2001; Dannenberg et al., 2000; Sag et al., 2000). The *INK4A* protein p16 inhibits the activation of cyclin-dependent kinases (CDKs). These enzymes stimulate progression of the cell cycle by phosphorylating the *RB* protein (pRB). As a result of the action of p16, however, unphosphorylated *RB* remains tightly bound to its major effectors, the E2F transcription factors, thereby preventing them from stimulating replication of DNA. In this way, p16 blocks entry of the cell into the S phase of the cell cycle. The current thinking is that inactivation of the *p53* and pRB tumor-suppressor pathways is essential for establishing tumorigenesis process in humans (Sherr et al., 2002). Also for this reason, oncogene-induced senescence has become the subject of intense research in order to identify new human oncogenes and tumor suppressor genes (Berns et al., 2004; Peeper et al., 2002).

Several recent reports provide an interesting link to the induction of senescence and the activation of the DNA damage response in small and early neoplastic human lesions

of various types, including dysplastic melanocytic nevi (Bartkova et al., 2005; Gorgoulis et al., 2005; Di Micco et al., 2006). Indeed, it has been long known that a senescence response also can result from DNA double-strand breaks (Di Leonardo et al., 1994). Members of the DNA damage sensors and effectors have been reported to be associated with cellular sensitivity to stress. For example, this is the case of ATM, a mutated gene in patients with ataxia-telangiectasia, which is a disorder characterized by chromosomal instability, hypersensitivity to γ -irradiation, predisposition to cancer, and premature senescence (Savitsky et al., 1995).

Collectively, these observations suggest that during tumorigenesis, before genomic instability emerges and results in multiple additional genetic aberrations, mammalian cells respond to inappropriate mitogenic signaling, DNA-replication stress or telomere dysfunction by activating protective cellular networks that abrogate cancer progression.

1.3.2 Melanoma and senescence

The first direct evidence of cellular senescence in a growth-arrested human neoplasm was reported for the melanocytic nevus (Michaloglou et al., 2005). In humans, cutaneous melanocytes reside largely in the epidermis and hair follicles. They are long-lived cells, despite exposure to potentially mutagenic insults such as UV radiation. Melanocytic nevi are exceedingly common and generally harbor an activating *BRAF*^{V600E} mutation or, less commonly, an *NRAS* or *HRAS* mutation (Pollock et al., 2003; Saldanha et al., 2004; Bastian et al., 2000). The *BRAF* mutation is also common in melanomas, where its silencing eliminates the transformed state (Hingorani et al., 2003; Davies et al., 2002). Indeed, mutant *BRAF* has attracted much attention as a potential therapeutic agent

in melanoma and other cancers (Tuveson et al., 2003). However, in spite of the activation of the MAPK pathway, which mediates proliferative signals, benign nevi eventually lose all their proliferative activity, and their growth remains arrested for decades, until they gradually disappear (Maldonado et al., 2004; Kuwata et al., 1993). Human melanocytic nevi that have reached the end of their growth phase display the four established hallmarks of oncogene-induced cell senescence: expression of an activated oncogene ($BRAF^{V600E}$), stable proliferative arrest, up-regulated levels of a tumor suppressor (p16) and elevated expression of senescence-associated acidic β -galactosidase. Of note, $BRAF^{V600E}$ can trigger the development of invasive melanoma only in the context of $p53$ deficiency, suggesting that $BRAF^{V600E}$ alone is insufficient to drive oncogenic transformation. Other model systems as well suggest that additional molecular defects are required for tumorigenesis mediated by $BRAF^{V600E}$ (Chudnokovsky et al., 2005). However, telomere length in congenital, acquired or Spitz nevi is not different from surrounding tissues (Miracco et al., 2002; Gray-Schopfer et al., 2006). These findings support the hypothesis of an active oncogene-driven senescence process in nevi (Figure 1.6), rather than senescence triggered by exhaustion of replicative potential resulting from telomere shortening. It is conceivable that non malignant cells, like melanocytes in nevi, upon the acquisition of oncogenic mutations may engage in three types of responses. Immediately, an antiproliferative response can be activated, leading to either programmed cell death (apoptosis) or senescence. Alternatively, cell proliferation driven by the oncogenic mutation may occur and produce a lesion. At this stage, both apoptosis and senescence programs might be activated. In the absence of appropriate defense mechanisms, additional genetic events may add to the continued growth leading to a

malignant lesion. At this stage, cells may undergo a senescence response and do so for decades, like appears to be the case for melanocytic nevi. Alternatively, cells may infrequently escape from such a senescent state and undergo malignant transformation, giving rise to melanoma progression (Figure 1.6).

Cutaneous melanocytes are exposed to mutagenic influences, are long-living and presumably cannot be replaced in large numbers. For this reason, there might exist evolutionary pressures tending to preserve them. Senescence might be a mechanism leading to this aim, since it represents a response to oncogenic stress that blocks proliferation but allows the cell to live on and perform its physiologic function.

However, although in principle the senescence response promotes survival by providing protection against cancer, it could interfere with individual long-term survival by promoting the accumulation of non proliferating cells. In fact, on one hand, senescence leads to a gradual accumulation of long-lived senescent cells, which can accumulate additional malignant alterations. On the other hand, senescent cells secrete matrix metalloproteinases and inflammatory cytokines that stimulate proliferation of preneoplastic epithelial cells thus contributing to the malignant transformation of nearby epithelial cells (Krtolica et al., 2001). Indeed, the beneficial anticancer effect of senescence process may result in negative consequences, which are manifested with advancing age (Campisi et al., 2005; Kirkwood et al., 2000).

1.3.3 Cellular senescence as a new anti-cancer therapy.

Many anticancer drugs are based on the induction of apoptosis. Apoptosis is mediated by a sequence of events that result in cell death. In cancer cells there is often an

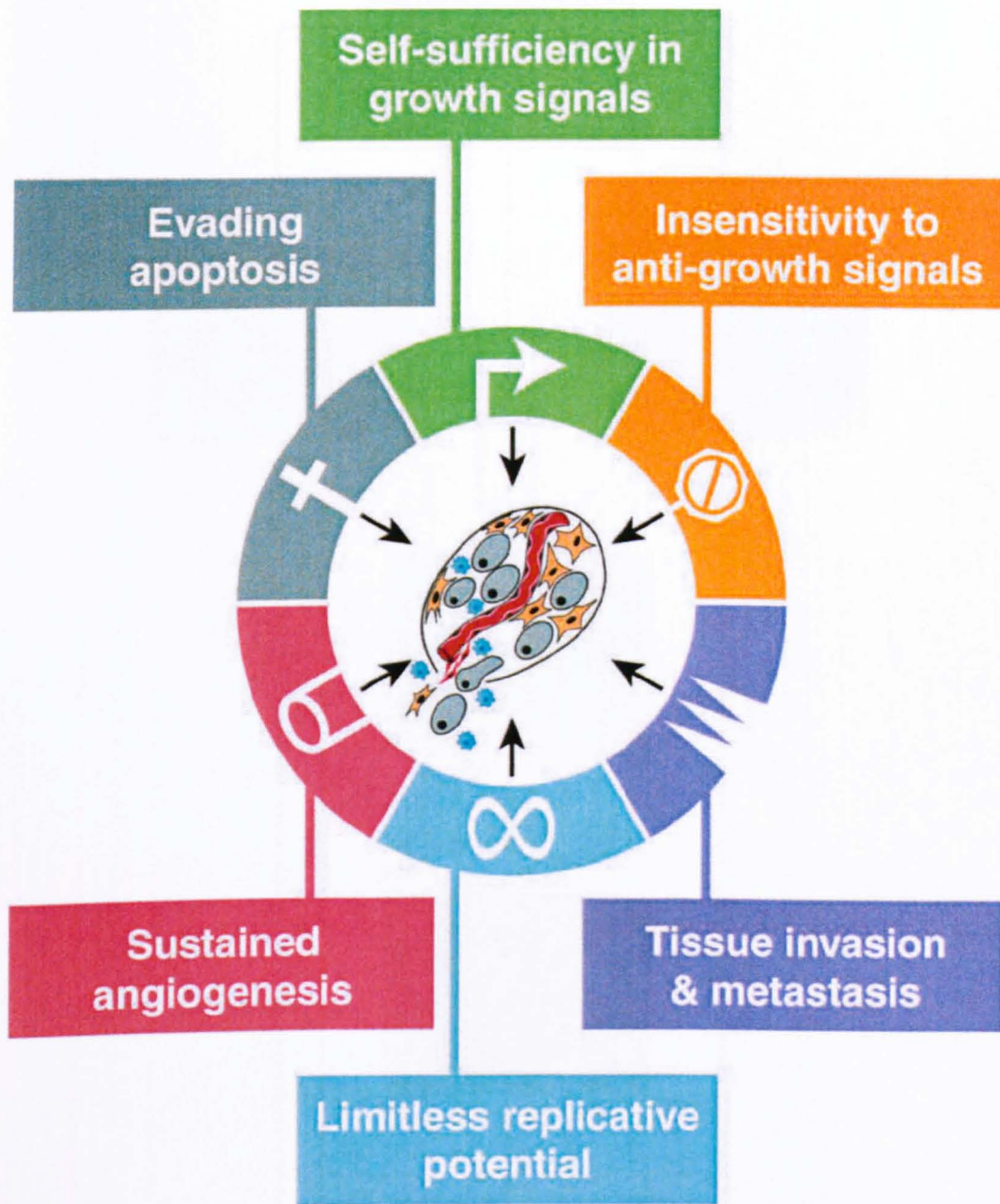
imbalance in favor of antiapoptotic signals, which confers cells a resistance to chemotherapeutic drugs and radiation. However, the intrinsic resistance of melanoma to the conventional pro-apoptotic chemotherapy has led researchers to evaluate new approaches.

In this contest, cellular senescence has emerged as an alternative target to overcome or even to prevent drug resistance from occurring. Alterations of senescence pathways without disruption of apoptosis may be sufficient to enhance chemotherapy efficacy. In cancer cells the capability to execute senescence might be disabled for numerous reasons. In fact, mutations in genes that control cellular senescence might have been acquired already during tumor development or may not be selected for during chemotherapy (Schmitt et al., 2002). Oncogenes such as activated RAS are known to provoke premature senescence relying on defects in this program (Serrano et al., 1997; Brookes et al., 2002). Nevertheless, tumors that preserve both an intact apoptotic and a functional senescence program may display a particularly robust drug response consisting of acutely inducible cell death in a first phase, corroborated by delayed apoptosis out of senescence at a later point. Although apoptosis is a fast-acting response mode, little is known about the possibility that apoptosis-competent cells might be sent into senescence following DNA-damaging therapy and whether a senescent tumor could ever undergo apoptosis upon an additional proapoptotic signal (Wang, 1995). Finally, senescence could be used as a mechanism to lock temporarily arrested tumor cells into irreversible cytostasis. So far, cytostasis, or growth arrest, has not been widely exploited for the treatment of malignant tumors. However, the rational design of anticancer compounds targeting selective entities unique to cancer cells has been adopted in an attempt to improve efficacy and selectivity,

and to minimize toxicity. The pharmacologic outcome of target-based compounds is more likely to be cytostatic than cytotoxic (Fox et al., 2002; Hoekstra et al., 2003). When considering growth arrest in chemotherapy, the irreversibility of this process is essential for therapeutic efficacy (Kahlem et al., 2004). The observation that cancer cells have maintained the ability to senesce *in vivo* has opened a new window of opportunity for cytostatic drugs in chemotherapy (te Poele et al., 2002). Replicative senescence, a permanent growth arrest, has been extensively studied in human diploid fibroblasts (Chang et al., 1999). Moreover, the induction of premature senescence was achieved both *in vitro* in tumor cell lines (Chang et al., 2002) and also *in vivo* following chemotherapeutic treatment it has been shown (te Poele et al., 2002). It has been suggested that the induction of senescence in breast cancer following adjuvant therapy is correlated to favorable outcome (te Poele et al., 2002). These results enforce the need of further investigations on the induction of senescence in cancer by compounds that target specific pathways.

For these reasons, further research needs to be directed towards a deep and clear understanding of how chemotherapeutic agents induce cellular senescence, and identification of drug targets that could be used in combination with chemotherapeutic drugs to facilitate irreversible growth arrest. The ultimate goal is to reach satisfactory levels of drug effectiveness with less toxic effects.

FIGURES OF CHAPTER 1



(From Hahnan D. et al., *Cell* 2000)

Figure 1.1. Hallmarks of cancer.

In the transformation process from normal to neoplastic phenotype, cell genotype acquires six essential alterations in the cell physiology.

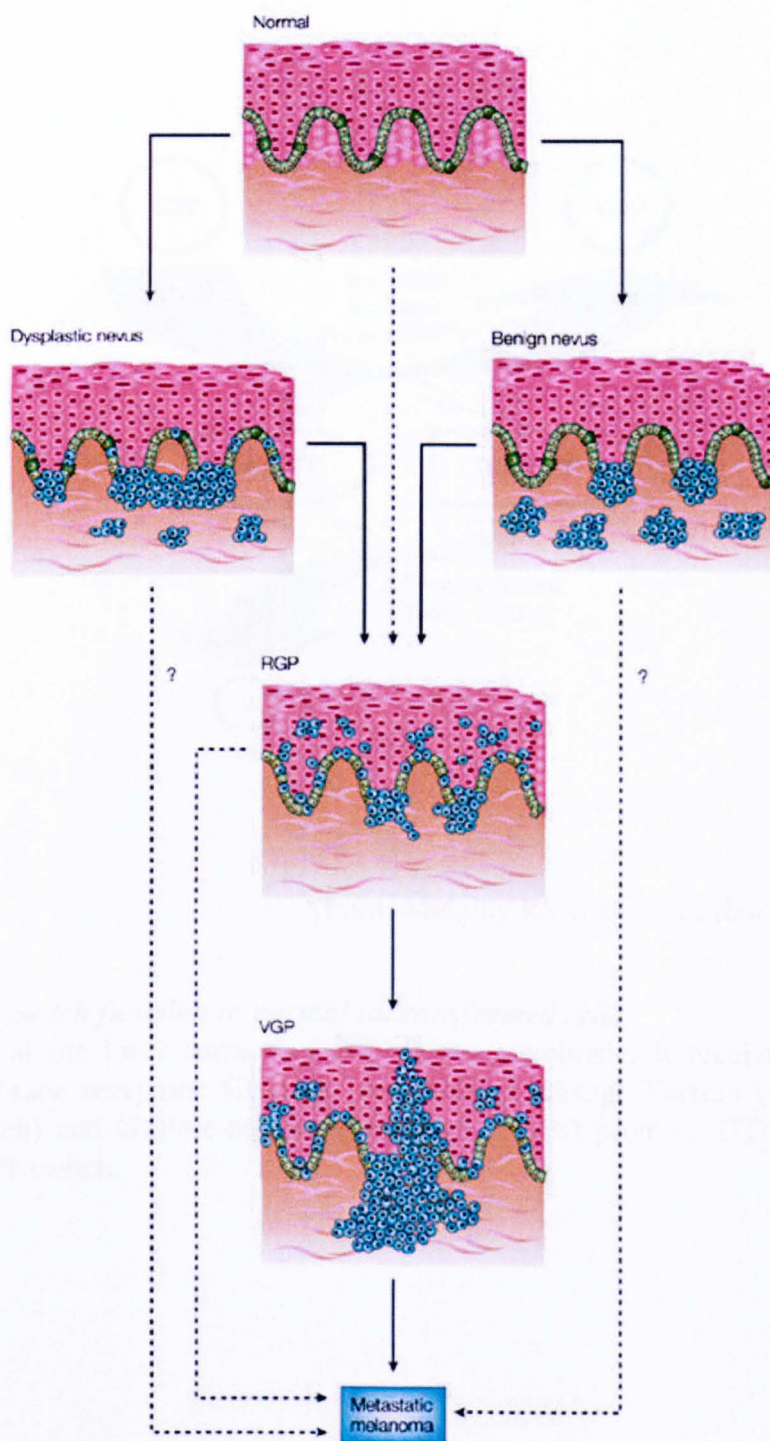
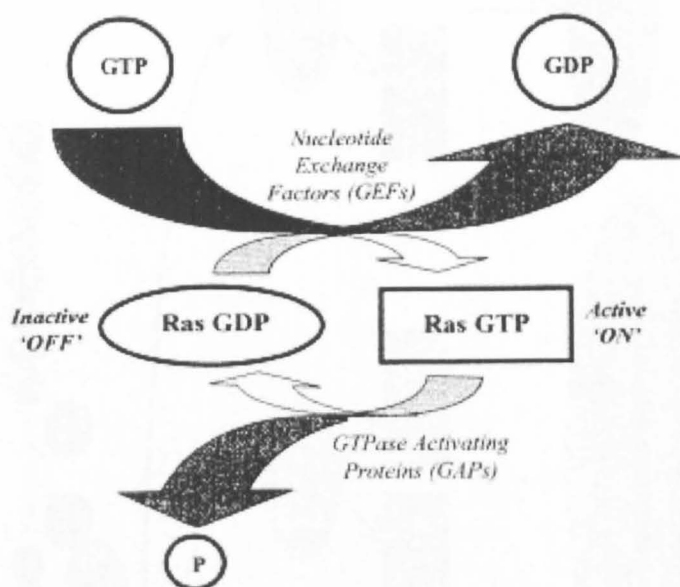


Figure 1.2. Melanoma histological stages.

Melanomas are histologically classified according to their location and stage of progression. Five distinct stages have been proposed in the evolution of melanoma on the basis of such histological criteria: common acquired and congenital nevi without dysplastic changes; dysplastic nevi with structural and architectural atypia; radial-growth phase (RGP) melanoma; vertical-growth phase (VGP) melanoma; and metastatic melanoma.



(From Midgley RS et al., *Crit. Rev. Oncol. Hematol.* 2002)

Figure 1.3. RAS switch function in normal untransformed cells.

RAS is situated at the inner surface of the plasma membrane. It receives input from ligand stimulated membrane receptors. Guanine Nucleotide Exchange Factors (GEFs) promote GTP binding (on-switch) and GTPase-activating-proteins (GAPs) promote GTP hydrolysis to GDP, constituting an off-switch.

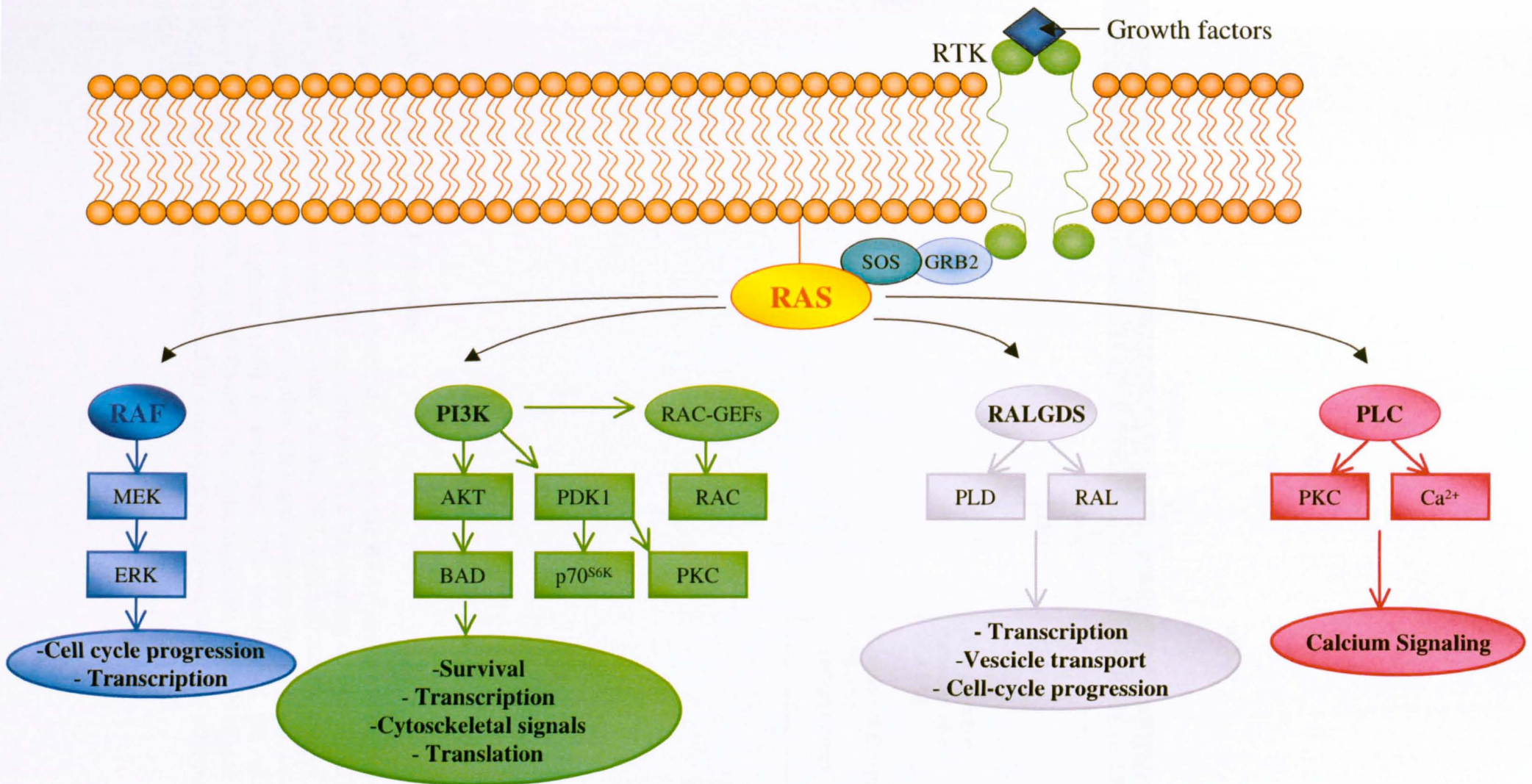


Figure 1.4: RAS controlled signaling pathways.

Growth factors bind to the Receptor Tyrosin Kinase (RTK) transmembrane proteins which activate downstream nucleotide exchange factors (GEFs) such as SOS. Upon GTP-bound state, RAS is active and transduces signal through different intra-cellular signaling pathways, such as RAF-MEK, PI3K-AKT, RALGDS, PLC-PKC pathways.

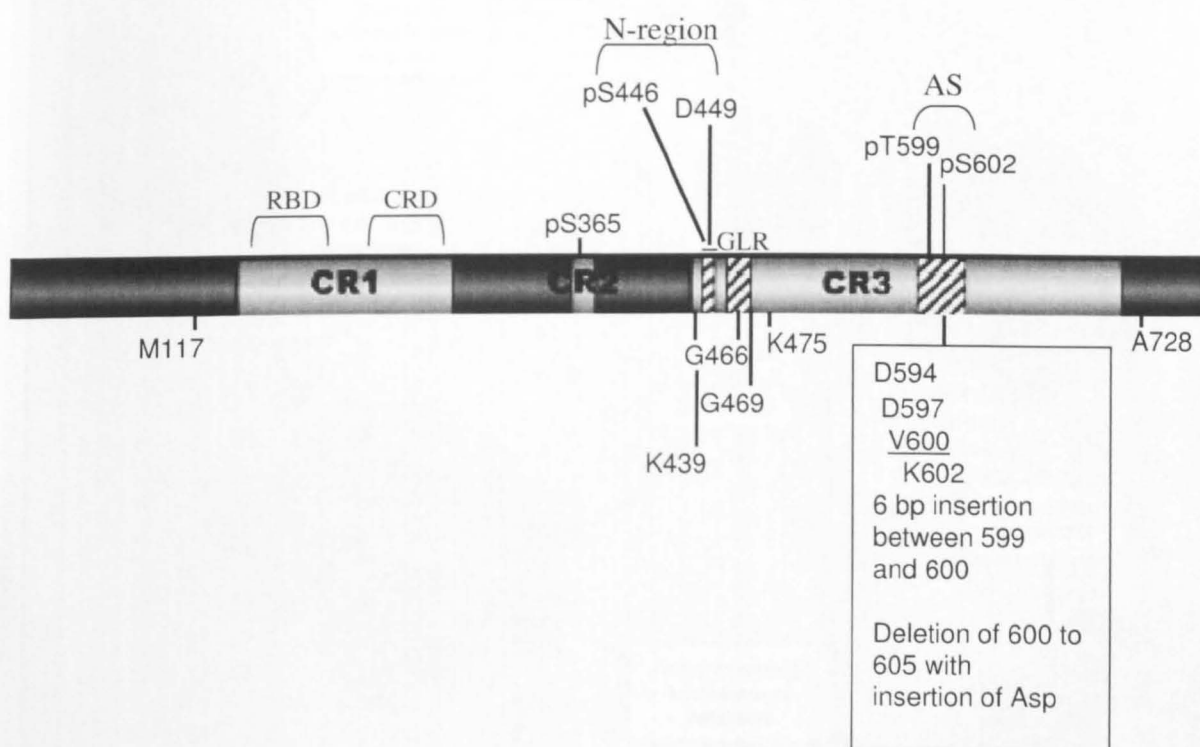
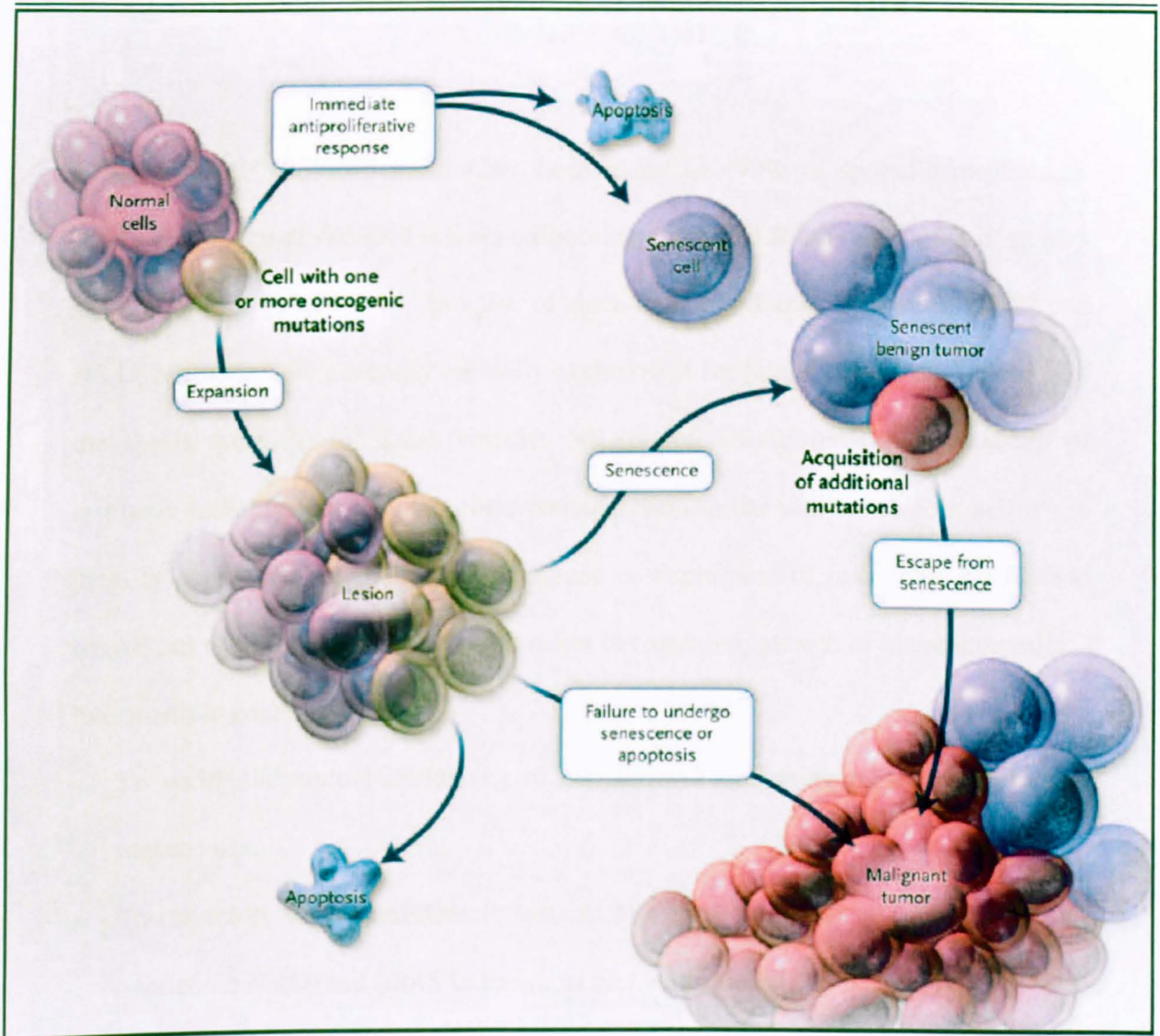


Figure 1.5: Structure of RAF proteins.

The RAF proteins share a common architecture and only the BRAF isoform is here depicted. The three conserved domains CR1, CR2 and CR3 are indicated. The Ras-binding domain (RBD) and cysteine-rich domain (CRD) within CR1 are indicated. S365, within CR2, which forms the core of a 14-3-3 binding site, is also shown. The kinase domain is within CR3 and contains the N-region, the glycine-rich loop (GLR) and the activation segment (AS) is indicated. The position of the sites of regulatory phosphorylation (pS466, pT599 and pS602) and one of the aspartates of the N-region are shown above the schematic. The sites that are mutated in melanoma are shown below the schematic; the common mutation V600, is underlined.



(From Mooi WJ et al., *N Engl J Med.* 2006)

Figure 1.6: A working model for tumor progression.

When it acquires an oncogenic mutation or mutations, a nonmalignant cell may engage in three types of response. An antiproliferative response can be activated, leading to either programmed cell death (apoptosis) or senescence. Alternatively, in the absence of an immediated response, the mutation-driven cell proliferation may produce a lesion. At this stage, both apoptosis and senescence programs might be activated or gain the upper hand, resulting in cell death or senescence. In the absence of appropriate response, they may do so for decades, which appears to be the case for melanocytic nevi. Cells may infrequently escape from such a senescent state and undergo malignant transformation.

AIMS OF THE THESIS

Mutated *BRAF* or *NRAS* genes have been found in ~90% of sporadic melanomas, with a frequency of ~65% of tumors harbouring a mutated *BRAF* and ~25% of tumors bearing an activated *NRAS*. In spite of their high frequency, activating *NRAS* and *BRAF* mutations are generally mutually exclusive at the single cell level. The aims of the thesis were: a) to assess whether *NRAS* and *BRAF* have a relationship of synthetic lethality that prevents their co-expression in the same melanoma cell; b) to identify genes that are modulated by forced co-expression of oncogenic *NRAS* and *BRAF* ; c) to evaluate the role of such genes in regulating growth of melanoma cells.

Intermediate goals:

- To verify the mutual exclusivity of *NRAS/BRAF* activating mutations in human melanomas.
- To develop an experimental system allowing simultaneous expression of oncogenic *NRAS* and *BRAF* in the same melanoma cells.
- To identify candidate genes which are significantly modulated when both oncogenic *NRAS* and *BRAF* are expressed in the same cell, compared to cells with single mutations, and which may mediate growth arrest in melanoma cells.
- To evaluate whether activators/inhibitors of previously identified genes can affect melanoma cell survival/proliferation.

II. MATERIALS AND METHODS

2.1 Melanoma cell lines.

Short-term melanoma cell lines used in this study were established as described previously (Anichini et al., 1996) from surgical specimens of lesions removed from patients admitted to our institute. Clinical-pathological features of melanoma specimens and the principal genetic alterations of the corresponding short-term cell lines have been previously described (Lupetti et al., 1994; Daniotti et al., 2004).

Melanoma clones from 665/1, 665/2, 665/R and 5810P were derived by two-layer soft-agarose technique as previously reported (Anichini et al., 1986). The obtainment of melanoma clones derived from 665/2/21 is described below (see paragraphs 2.5 and 2.6).

All cell lines were cultured at 37°C in a humidified 7.5% CO₂ atmosphere and routinely tested for the absence of Mycoplasma contamination by the Mycoplasma Plus PCR primer set kit (Stratagene; La Jolla, CA). Melanoma cells were routinely grown in medium RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with either 10% FCS (Biowhittaker- for all melanoma lines except the 665/2/21- derived clones) or FBS (BD Biosciences, Clontech, San Jose, CA- for the 665/2/21-derived clones), 2 mM L-glutamine (BioWhittaker), 20 mM HEPES buffer (BioWhittaker), 200 units/mL penicillin (Pharmacia, Milan, Italy) and 40 µg/mL Gentilyn (Italfarmaco, Milan, Italy). For fibroblasts the same culture conditions were applied, but DMEM (BioWhittaker) medium was used instead of RPMI. Melanocytes were cultured in Melanocyte Growth Medium M2 (PromoCell, Heidelberg, Germany) and 5% CO₂ atmosphere was used for cell maintenance.

Matrigel cultures were performed by coating dishes with BD Matrigel Basement Membrane Matrix (BD Biosciences, Clontech) following the manufacturer's instructions.

2.2 Mutant-allele-specific amplification (MASA)-PCR.

MASA-PCR for the detection of *BRAF*^{V600E} and *NRAS*^{Q61R} was conducted as previously described (Xu et al., 2002; Linard et al., 2002) on genomic DNA isolated by standard methods from melanoma cells. Specific forward primers #1-#2 (Table 2.1) carrying a 3'-end two bases substitution were used to amplify selectively mutant *NRAS* or *BRAF* genes respectively, as previously described (Xu et al., 2002; Linard et al., 2002). Forward primers #3-#4 were used as positive control to amplify wild type as well as mutant *NRAS* exon 3 or *BRAF* exon 15 (Table 2.1). Reverse primers used for all PCR were #5-#6 (Table 2.1). Amplifications were performed in a 25- μ l PCR reaction mixture containing 2.5 mM of each dNTP, 0.1 μ M of each primer, 0.6U of HotMaster Taq DNA polymerase (Eppendorf, Hamburg, Germany) and 100 ng/sample of genomic DNA template. The PCR reaction was set with an initial denaturation of 3 min at 94°C followed by 35 cycles of template denaturation for 20 sec at 94°, primer annealing at 55° for 15 sec, primer extension at 65° for 30 sec and by a final elongation at 65° for 1 min. PCR product expected length was confirmed by a 1.5% TAE agarose gel electrophoresis (129 bp for *BRAF*^{V600E}, 215 bp for *BRAF*^{wt}, 106 bp for *NRAS*^{Q61R} and 106 bp for *NRAS*^{wt}). Samples resulted negative by 35 cycles MASA amplification were tested in the same conditions with 45 amplification cycles.

2.3 Sequence analysis of BRAF exon 15, NRAS exon 3 and plasmid constructs.

Amplification of *BRAF* exon 15 and *NRAS* exon 3 was performed by primers #7 to #10 (Table 2.2) (Davies H. et al., 2002) on genomic DNA isolated by standard methods from melanoma cells. *BRAF* and *NRAS* mutations were detected by direct sequencing of the PCR products.

Primers for pTRE-based plasmids, mapping on the pTRE2hyg- response plasmid (see paragraph 2.6), were purchased by BD Biosciences, Clontech.

All amplified products were gel purified (NucleoSpin Extract, Machinery-Nagel, Düren, Germany) and automatically sequenced using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, CA). Sequencing primers for *BRAF* exon 15, *NRAS* exon 3 and full-length cDNA *NRAS* were #11-#16 (Table 2.3).

2.4 Construction of constitutive and inducible expression plasmids for *NRAS*^{WT} and *NRAS*^{Q61R}.

Wild type human *NRAS*, amplified with primer #17-#18 (Table 2.4), was cloned by PCR into pcDNA3.1/V5/His plasmid using the Eukaryotic TOPO TA cloning Kit (Invitrogen, Carlsbad, CA) and sequenced to verify correspondence to the official *NRAS* cDNA sequence (RefSeq: NM_002524). The resulting constitutive expression vector (pcDNA3.1-Flag*NRAS*^{WT}) was then digested with Bam H1 and Not I restriction enzymes and the recovered fragment was cloned into BamH1/NotI digested response plasmid pTRE2hyg- (BD Biosciences, Clontech) to obtain pTRE2hyg-Flag*NRAS*^{WT}. pcDNA3.1-Flag*NRAS*^{WT} and pTRE2hyg-Flag*NRAS*^{WT} were then subjected to site-directed mutagenesis by the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla,

CA) using primers #19 and #20 (Table 2.5). This yielded pcDNA3.1-Flag*NRAS*^{Q61R} and pTRE2hyg-Flag*NRAS*^{Q61R} plasmids containing the Q61R mutation, as result of an A/G substitution. The single nucleotide coding change A182G in both plasmids was confirmed by sequencing.

2.5 Generation of a melanoma clone constitutively expressing *NRAS*^{Q61R}.

21 cells were transfected with pcDNA3.1-Flag*NRAS*^{Q61R} plasmid and selected for two weeks in complete medium containing G418 (0.8 mg/mL) (Sigma-Aldrich, St. Louis, MO). DNA was extracted from each of the selected colonies, with lysis buffer containing 50 mM Tris pH 8.5, 1 mM EDTA and 0.5% Tween-20 and amplified with GenomiPhi enzyme (Amersham Bioscience, Buckinghamshire, United Kingdom). Presence of Flag*NRAS*^{Q61R} insert was tested by PCR, and Q61R mutation was detected by direct sequencing. A stable selected clone was named 21*NRAS*^{Q61R}.

2.6 Generation of melanoma clones with inducible expression of mutated or wild type *NRAS*.

The Tet-Off System (Clontech Laboratories, Palo Alto, CA) includes the tetracycline-controlled regulatory pTet-Off vector, the pTRE2hyg- response vector as well as the pTRE2hyg-*Luc* control vector (Figure 2.1). Hygromycin B (Hyg) and Doxycycline (Dox) were both purchased from Clontech. FuGENETM 6 (Roche Molecular Biochemicals, Mannheim, Germany) was used for all transfection experiments, according to the manufacturer's instructions. 21 cells were transfected with pTet-Off vector and clones were isolated as described for the constitutive transfection. Each clone was splitted

into two cultures in a 24-well plate and transfected with pTRE2hyg-*Luc* in presence or absence of Dox (1 µg/mL). Following a 48 hour incubation at 37°C, cells were harvested and lysed by Glo Lysis Buffer (Promega Corporation, Madison, WI). Steady Reagent (Promega) was added to cell lysates and Relative Light Units (RLU) were measured using a plate reader luminescence spectrophotometer (TECAN ULTRA 384, Gentronix, Manchester, UK). The RLU (-Dox)/ RLU (+Dox) ratio was calculated for each well. The clone displaying the highest RLU ratio, named 21Off, was selected as stable pTet-Off expressing recipient line for the second step of transfection. To this end, pTRE2hyg-Flag*NRAS*^{WT} or pTRE2hyg-Flag*NRAS*^{Q61R} plasmids were independently transfected into 21Off and stable clones were selected with G418 (0.5 mg/mL) and Hyg (0.25 mg/mL) for two weeks. Antibiotic-resistant stable transfectants were propagated and maintained in the same selective medium. The resulting cell lines were tested for *NRAS*^{Q61R} mutation by PCR.

2.7 Immunoblotting.

Lysates were prepared from melanoma cells and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions as described (Zanon et al., 2004). Proteins were transferred onto PVDF membranes (Hybond-P, Amersham) by standard procedures. Membrane blocking in nonfat milk and incubation with primary antibodies were as described (Zanon et al., 2004). Primary antibodies were: anti-*NRAS* (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-actin (1:2000) (Sigma-Aldrich), anti-ERK (1:5000) (Sigma-Aldrich), anti-activated MAPkinase (1:10000) (Sigma-Aldrich); anti-phospho-AMPK-α (Thr172) (1:1000) (Cell Signaling Technology,

Danvers, MA); anti-p21 (BD Biosciences, PharMingen). Membrane washing and incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG or goat anti-rabbit IgG) were carried out as described (Zanon et al., 2004). Development was done by the chemiluminescence method with the SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL) and autoradiography.

2.8 Ras Activation Assay.

Activation state of NRAS was assessed by the RAS Activation Kit (Stressgen, San Diego, CA) according to the manufacturer's instruction. This assay uses a peptide corresponding to the RAS-binding domain of BRAF as a RAS-GTP-dependent probe to immunoprecipitate active NRAS from cell lysates (de Rooij and Bos, 1997). The immunoprecipitated active RAS was then detected by Western Blot analysis using the specific antibody included in the kit.

2.9 Proliferation assay.

Cells were seeded in a 96-multiwell plate (Corning Inc., Corning, NY) in their own selective medium with or without addition of Dox (1 µg/mL). A freshly prepared solution of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-Tetrazolium inner salt (MTS)/phenazine methosulfate (PMS) (ratio 20/1) was added (20 µL) to each well every 24 hr, between 24 and 96 hours after beginning of the cell culture. Colorimetric evaluation of the conversion of MTS into a soluble formazan

product was carried out by reading absorbance at 490 nm with a BioRad 550 plate reader (BioRad, Hercules, CA).

2.10 Senescence assay.

Cells were cultured in their own selective medium with or without addition of Dox (1 $\mu\text{g/mL}$) in a 12-well culture plate (Corning Inc.). Cells were fixed at 70% confluence and then incubated at 37°C overnight with the staining solution containing the X-gal substrate (Senescence Detection Kit, MBL International Corporation, Woburn, MA). Cells were then observed under a microscope for development of blue color.

2.11 Cell cycle analysis.

Cell cycle analysis was carried out in cultures at 50-60% confluency. To this end, cells were detached by 3 min trypsin (500 mg/L) (BioWhittaker) treatment, resuspended in cold EtOH 70% and kept in ice for 20 minutes. After washing in Vindelov Solution (3.4 mM NaCitrate, 0.01% NP4, 1.5 mM Spermina Tetrahydrochloride, 0.5 mM Tris, 10 $\mu\text{g/mL}$ RNA-ase), cells were stained with Propidium Iodide (Sigma-Aldrich). Cell cycle analysis was carried out by a FACScalibur flow cytometer (BD Biosciences) and phases of the cell cycle were evaluated by the ModFit Software (BD Biosciences).

2.12 Expression of cell surface and intra-cellular antigens by flow cytometry.

Expression of cell surface or intra-cellular antigens (in saponin permeabilized cells) was carried out as previously described (Maccalli et al., 1999). mAbs to Delta (SY-5), MB1 (SJJ-3), LMP2 (SY-1), LMP7 (SY-3), LMP10 (TO-7), Tapasin (TO-3),

Calnexin (TO-5), Calreticulin (TO-11), ERp57 (TO-2), MICB (SJJ-5) and β 2-microglobulin (L368) have been described elsewhere (Bandoh et al., 2005; Ogino et al., 2003; Lampson et al., 1983). The TAP1-specific mAb NOB-1 and the TAP2-specific mAb NOB-2 are secreted by hybridomas derived from the fusion of murine myeloma cells P3-X63-Ag8.653 with splenocytes from BALB/c mice immunized with partial length TAP1 recombinant protein (434-735) and a keyhole limpet hemocyanin (KLH)-conjugated TAP1 peptide (717-735) and with partial length TAP2 recombinant protein (316-703), respectively, by the strategy described elsewhere (Ogino et al., 2003). Staining for cell surface antigens was performed with mAbs directed to HLA-class I (W6/32) (Parham et al., 1979), HLA-DR, HLA-DQ, HLA-DP, integrin- β 1, - β 4 (all from BD Biosciences, PharMingen), integrin- α 3, - α v β 3, VLA-2, VLA-4, VLA-5, VLA-6, ICAM-1, and LFA-3 (all from Chemicon International, Temecula, CA). Cells were then stained with a fluorescein-conjugated affinity purified goat anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA) and analyzed with a FACSCalibur cytofluorimeter (BD Biosciences).

2.13 Cell mediated cytotoxicity and limiting dilution analysis of cytotoxic effector frequency.

Non specific lymphokine activated killer (LAK) cells were generated by culturing peripheral blood lymphocytes (PBL) of healthy donors in RPMI 1640 -10% pooled human serum with 1% PHA (Murex Biotech Ltd., Temple Hill, DA) for three days and then with IL-2 at 3000 IU/mL (Chiron, Emeryville, CA) for two weeks. Lysis of targets by LAK cells was evaluated by a 4-h ^{51}Cr release assay at E:T cell ratios from 100:1 to

1.5:1, as described previously (Anichini et al., 1999). A T cell line was generated by culturing PBL from a HLA-mismatched healthy donor for 3 weeks in mixed lymphocytes-tumor culture (MLTC) with irradiated (30,000 cGy) melanoma transfectants bearing a silenced NRAS^{Q61R}, in RPMI 1640 medium supplemented with 300 IU/mL IL-2 and 10% pooled human serum. The culture was re-stimulated weekly. The resulting T cell line was used in a split well, limiting dilution assay, as described (Anichini et al., 1999). The HLA-class I antigen-restriction of the lysis was assessed by measuring its inhibition by the HLA-class I antigen-specific mAb W6/32, as described (Anichini et al., 1999). The threshold of significant lysis, criteria to score a well containing HLA class I antigen-restricted T cells and non-restricted cytotoxic effectors, and data analysis for frequency determination have been described elsewhere (Anichini et al., 1999).

2.14 Gene expression profiling with CodeLink system and statistical data analysis.

Total RNA was extracted from melanoma cell lines using the TriPure Isolation Reagent (Roche Molecular Biochemicals, Mannheim, Germany) and its quantity and quality were assessed by UV spectrophotometry and gel electrophoresis. In order to analyze differential gene expression levels of the samples, the CodeLink Human Whole Genome Bioarrays (GE Healthcare, Amersham Bioscience, Buckinghamshire, United Kingdom) were used. Slide hybridization and processing were performed following manufacturer's instructions. Briefly, cDNA was prepared starting from 2 µg of total RNA; then, cRNA was synthesized by *in vitro* transcription using a single labeled nucleotide, biotin-11-UTP, in the IVT reaction at a concentration of 1.25 mM. Unlabeled UTP was present at 3.75 mM, while GTP, ATP and CTP were at 5 mM. The mixture was

incubated at 37°C overnight for 14 hours. The labeled cRNA was then purified using the RNeasy mini kit (Qiagen, Hilden, Germany). Then 10 µg of purified cRNA was fragmented in 1X fragmentation buffer (CodeLink Expression Assay Reagent Kit, Amersham) at 94°C for 20 minutes. For hybridization with CodeLink bioarrays, 10 µg of fragmented cRNA in 260 µL of hybridization solution was added to each bioarray and incubated for 18 hours at 37°C while shaking at 300 r.p.m. After washing, slides were stained with Cy5-streptavidin (Amersham Biosciences) and scanned using a GenePix 4000B scanner (Axon Instruments, Downington, PA). The scanned image files were analyzed using CodeLink software and GeneSpring 7.2 software (SiliconGenetics, Agilent, Santa Clara, CA). Data analysis was performed in collaboration with and under the supervision of Dr. Ewan Hunter from SiliconGenetics. For each spot the raw intensity was calculated by the CodeLink software as the difference between the spot mean and the local background mean. Then raw hybridization signals were normalized on each spot as ratios to the median of the array for better cross array comparison. These values were then further *Per Chip* normalized by GeneSpring and a Quality Check (QC) on normalized data was performed. QC-passed genes were then analyzed by Anova one-way parametric test with none correction (T-test: 0.01). For data mining, this data set was used in the *BiNGO* software, an open-source Java tool to determine which Gene Ontology (GO) terms are significantly overrepresented in a set of genes, and a subsequent statistical analysis was performed in order to classify the list of modulated genes between the two samples analyzed.

2.15 Microarray data validation by RealTime PCR.

The Assays-on-Demand Gene Expression Products (Applied Biosystems, Foster City, CA) with TaqMan FAM dye-labeled probes were used for the detection and quantification of specific human genetic sequences in RNA samples converted to cDNA. Gene expression quantification was performed as the second step in a two step-reverse transcription-polymerase chain reaction (RT-PCR). Reactions (20 μ L) contained 10 ng of total RNA diluted in RNase-free water, 10 μ L 2X TaqMan Master Mix, 1 μ L Assays-on-Demand Gene Expression Assay Mix. The assays utilized are listed below in Table 2.6. Each amplification was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) for 10 min at 95°C followed by 40 cycles of denaturation (15 sec at 95°C) and annealing/extension (1 min 60°C). Data were analyzed using ABI PRISM Sequence Detection Software (SDS) version 2.1 and then further processed using Microsoft Excel. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the endogenous control for the normalization of input target RNA. The comparative or relative $\Delta\Delta C_T$ method was used for quantification of target amounts. The $\Delta\Delta C_T$ value represents the difference between the average ΔC_T value of NRAS^{61ON} and the average ΔC_T value of NRAS^{61OFF}, where ΔC_T is the difference between the threshold cycle (C_T) value of each target gene and the C_T value of the corresponding endogenous reference gene (GAPDH).

Expression levels of LOXL3 protein were further validated using a monoclonal mouse antibody against LOXL3 (1:1000) (Abnova, Taipi City, Taiwan) by Western Blot, as previously described.

2.16 Silencing of LCN2 by siRNA.

Melanoma cells were seeded in 6-well tissue culture plates (Corning Inc.) and after 24 hours, when cell density was about 80%, were transfected with LCN2-specific small interfering RNA (siRNA) (Dharmacon, Lafayette, CO), using Lipofectamine (Invitrogen). Either pEGFP vector or a Luciferase-siRNA were also transfected respectively as positive control of the transfection procedure or as negative control for selective silencing of LCN2 RNA. Silenced expression of intra-cellular LCN2 protein was in saponin permeabilized cells was validated using a monoclonal mouse antibody against LCN2 (AbCam, Cambridge, UK) in flow-cytometry analysis as previously described (Maccalli et al., 1999).

2.17 Rapid lysis of cultured cells for AMPK kinase assay and for Western Blot.

Melanoma cells, fibroblasts and melanocytes were seeded in 6 cm culture dishes and grown until nearly confluent in their own medium with or without AICAR or Phenformin treatment. Cells were lysed as previously described (Hardie et al, 2000). Briefly, the culture medium was rapidly removed and replaced with ice-cold PBS, and the plates were placed on ice. Then the PBS was poured off, the residual medium removed with a Pasteur pipette and 0.2 mL of ice-cold cell lysis buffer (50 mM Tris/HCl, pH 7.4 at 4°C, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 250 mM mannitol, 1% (v/v) Triton X-100, 1 mM DTT, 1 mM benzamidine, 0.1 mM phenylmethane sulfonyl fluoride (PMSF) 5µg/mL soybean trypsin inhibitor (SBTI)) was added to each plate. The cells were scraped from the dish, the lysate was centrifuged

(12,900xg, 4°C, 3 min) and the supernatant was snap-frozen in liquid N₂ and stored at -80°C until use.

2.18 Assay of AMPK kinase activity.

Kinase activity of AMPK in melanoma samples was tested as previously described (Davies et al., 1989). Briefly, AMPK was immunoprecipitated from 200 µg of cell lysate using 10 µg of anti-subunit α 1 and 10 µg of anti-subunit α 2 affinity purified sheep anti-AMPK polyclonal antibodies (Woods et al, 1996) in AMPK immunoprecipitation (IP) Buffer (50 mM Tris/HCl, pH 7.4 at 4°C, 150 mM NaCl, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 0.1 mM phenylmethane sulfonyl fluoride (PMSF), 5 µg/mL soybean trypsin inhibitor (SBTI)) for 1 hour at 4°C. Immunocomplexes were washed with IP buffer plus 1 M NaCl and then with Hepes Assay Buffer (50 mM Na Hepes, pH 7.0, 1 mM DTT, 0.02% Brij-35). AMPK activity in immunocomplexes was determined by phosphorylation of AMARA substrate peptide (AMARAASAAALARRR) with ATP mix (200 µM ³²PATP/5 mM MgCl₂, 200 µM AMARA peptide, 1 mM AMP). After 10 min at 30°C, the reactions were terminated by spotting 30 µL aliquots onto Whatman P81 filter paper, followed by 2 rinses in 1% (v/v) phosphoric acid with gentle stirring to remove free ATP and membranes were then dried. Phosphorylated substrates were measured by scintillation counting. Calculation of Units of enzyme activity (U) per mg of protein was carried out as previously described (Fryer et al., 2002).

2.19 Detection of western blots by infra-red imaging.

Phosphorylation of either the AMPK α -subunits in Thr172 (pAMPK) or acetyl CoA-carboxylase in Ser79 (pACC) was evaluated in Western blot by using phospho-specific antibody from respectively Cell Signaling Technology or kindly gifted from D.G. Hardie, (University of Dundee, Dundee, Scotland), as previously described (Chen et al., 2000). Precast Bis-Tris 4–12% gradient polyacrylamide gels (Invitrogen) for both phosphorylated and total AMPK, and pre-cast 3–8% Tris-acetate gels (Invitrogen) for both phosphorylated and total acetyl-CoA carboxylase, were used. Proteins were transferred onto nitrocellulose membranes (BioRad, Hemel Hempstead, UK) using the Xcell II Blot Module (Invitrogen). To analyze phosphorylation levels of ACC, membranes were incubated in LI-COR Odyssey™ Blocking buffer (LI-COR Biosciences, Nebraska, USA) for 1 hour. Anti-pACC antibody (1.46 $\mu\text{g/mL}$ in blocking buffer containing Tween-20 0.2% v/v) was then added and left shaking for 1 hour. The membranes were washed 6 times for 5 min with TBS (10 mM Tris-HCl, pH 7.4, 0.5 M NaCl) plus Tween-20 (0.2% v/v) (TTBS). The membranes were immersed in blocking buffer containing Tween-20 (0.2% v/v) and 1 $\mu\text{g/mL}$ anti-sheep antibody conjugated to IR dye 680 (Molecular Probes, Leiden, The Netherlands) for the detection of pACC and 1 $\mu\text{g/mL}$ streptavidin conjugated to IR Dye 800 (Rockland Inc., Lorne Laboratories, Reading, UK) for ACC and left shaking for 1 hour, protected from light. The membranes were then washed 6 times for 5 min using TTBS and 1 time for 5 min in distilled H_2O . The membranes were scanned in two different channels using the Odyssey IR imager (LI-COR Biosciences), the results quantified using Odyssey software (LI-COR Biosciences) and expressed as a ratio of the signal obtained with the pACC antibody to that obtained

with streptavidin. Analysis of phosphorylation of AMPK was similar except that the 4–12% Bis-Tris gels were used, and the membranes were simultaneously probed for 1 hour with the anti-pAMPK and anti- $\alpha 1$ and $\alpha 2$ antibodies (Cell Signaling), followed by the secondary anti-rabbit IR Dye 680 and anti-sheep IT Dye800 antibodies respectively (both Rockland Inc.)

2.20 Evaluation of Apoptosis.

For the evaluation of apoptosis, 1×10^6 melanoma cells were seeded in 6 cm diameter plates (Corning Inc.) in their own medium; after 24 hours, culture medium was substituted with the same medium with or without different doses of AICAR or Phenformin and cells were maintained for 8 days. Quantification of apoptotic cells was done by flow cytometry with a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA) using the Annexin V-FITC kit (BD Biosciences, PharMingen, San Diego, CA) as previously described (Salvucci O et al., 2001).

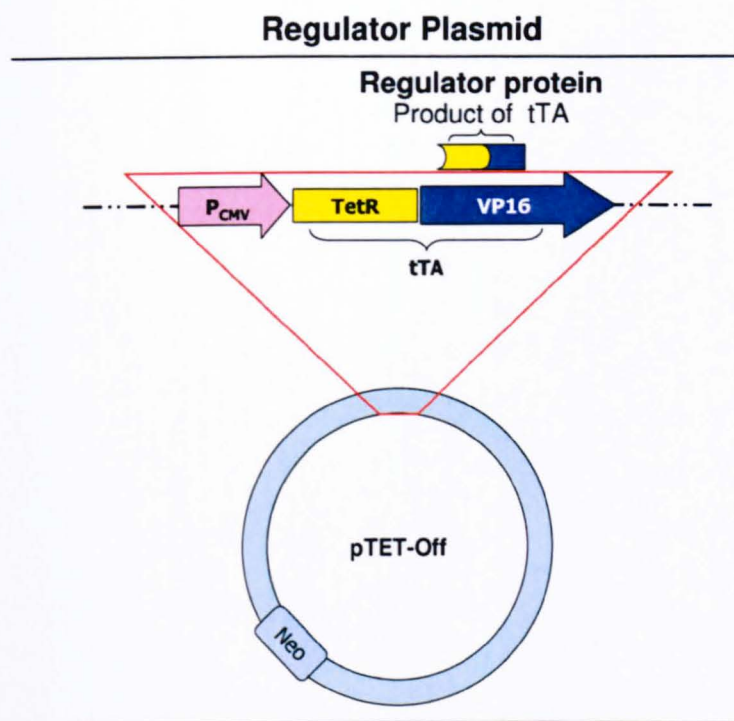
2.21 Flow-cytometric analysis of β -galactosidase expression.

In order to measure and quantify β -galactosidase activity in melanoma cells, fluorescein digalactoside reagent was used. Fluorescein di- β -D-galactopyranoside (FDG) is a fluorogenic substrate for β -galactosidase (Sundararajan, 1963). Nonfluorescent FDG is sequentially hydrolyzed by the enzyme, first to fluorescein monogalactoside (FMG) and then to highly fluorescent fluorescein (Fieldler et al., 1994).

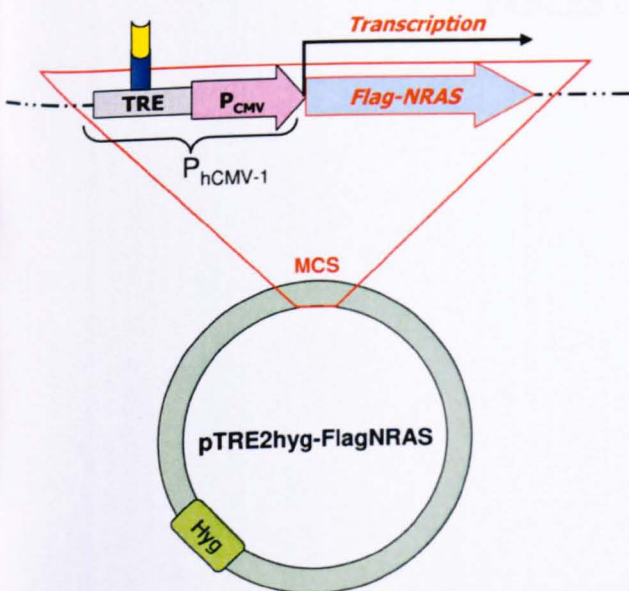
Melanoma cells were seeded in 60 mm plates (Corning Inc.) and after 24 hours were treated or not with 1 mM AICAR or Phenformin. On the 11st day, cells were trypsinized

and cell suspension in growth medium was mixed with an equal volume of FDG solution and then incubated at 37°C for 1 minute. The suspension was then diluted 10-fold with ice-cold growth medium and maintained at 4°C on ice for 15 minutes. Before analysis, each sample was stained with 5 μ M propidium iodide to identify those cells that did not survive the procedure. Fluorescence levels were read by flow cytometry in a FACScalibur cytofluorimeter (BD Biosciences) and data analyzed by Winmdi software.

FIGURES OF CHAPTER 2



In the absence of Dox



In the presence of Dox **D**

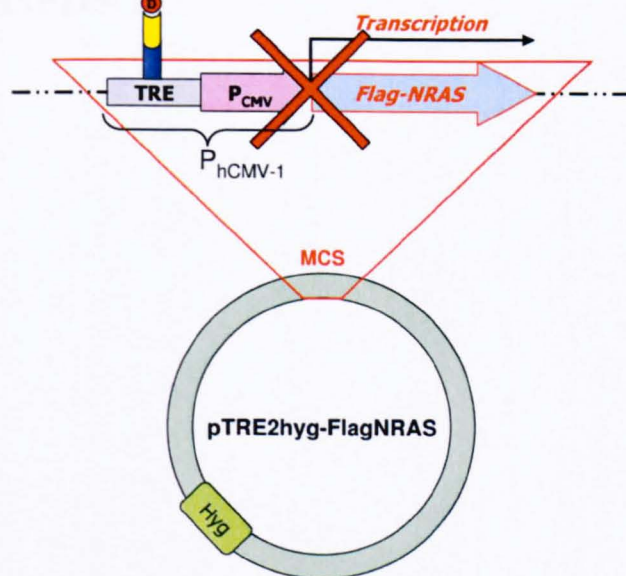


Figure 2.1. Schematic illustrative diagram of the Tet-Off system used in the study.

Regulator plasmid pTet-Off encodes the regulator protein that binds to the TRE sequence in the response plasmid. In the absence of Dox, regulator protein binding to TRE activates transcription of the *Flag-NRAS* construct. In the presence of Dox, the antibiotic forms a complex with the regulator protein blocking transcription of the *Flag-NRAS* construct. P_{CMV} : early promoter of cytomegalovirus; tTA: tet-responsive transcriptional activator; TetR: tet repressor; VP16: C-terminal activation domain of the VP16 protein of herpes simplex virus; TRE: tet-responsive element; Dox: doxycyclin; Neo: neomycin resistance gene; Hyg: hygromycin resistance gene; P_{hCMV-1} : tet-responsive promoter.

TABLES OF CHAPTER 2

	<i>BRAF</i> ^{V600E}	<i>NRAS</i> ^{Q61R}
Specific	#1:GGTGATTTTGGTCTAGCTACAAA	#2: ATACTGGATACAGCTGGAAG
Control	#3:TAGGTGATTTTGGTCTAGCTACAGT	#4:CATACTGGATACAGCTGGAC
Reverse	#5: GGCCAAAAATTTAATCAGTGGA	#6: TGACTTGCTATTATTGATGG

Table 2.1. Primer for MASA-PCR of *NRAS*^{Q61R} and *BRAF*^{V600E} (Xu et al., 2002; Linard et al., 2002).

	F (5'→ 3')	R (5'→ 3')
<i>BRAF</i> exon 15	#7: AACACATTTCAAGCCCCAAA	#8 : AGCATCTCAGGGCCAAAAAT
<i>NRAS</i> exon 3	#9: GATTCTTACAGAAACAAGT	#10: AACCTAAAACCAACTCTTCCCA

Table 2.2. Primer for the amplification of *BRAF* exon 15 (#7-#8) and *NRAS* exon 3 (#9-#10) on genomic DNA.

F: forward, R: reverse.

	F	R
<i>BRAF</i> exon 15	#11:TCATAATGCTTGCTCTGATAGGA	#12: GGCCAAAAATTTAATCAGTGGA
<i>NRAS</i> exon 3	#13: GGTGAAACCTGTTTGTTGGA	#14: ATGACTTGCTATTATTGATGG
<i>NRAS</i> full-length	#15: TAATACGACTCACTATAGGG	#16: TAGAAGGCACAGTCGAGG

Table 2.3. Primer for sequencing of *BRAF* exon 15 (#11-#12), *NRAS* exon 3 (#13-#14) and full length cDNA *NRAS* (#15-#16).

F: forward, R: reverse.

F	#17: <u>GCCACCATGGACTATAAGGATGACGATGAC</u> ACTGAGTACAAACTGGTGGTGGTTGGA
R	#18: TTACATCACCACACATGG

Table 2.4. *Primer used for amplification of full-length cDNA NRAS.*

Forward primer (F) contained a Kozak consensus sequence (underlined) with an ATG start codon and a minimal N-terminal tag (*italic bold*) encoding for the Flag epitope F7 (DYKDDDD), described (Johnson et al., 2002) as not interfering with RAS functions and stability; reverse (R) primer contained the stop codon (**bold**).

F	#19: CTGGATACAGCTGGAC G AGAAGAGTACAGTGCC
R	#20: GGCACTGTACTCTTCT C GTCCAGCTGTATCCAG

Table 2.5. *Primer for achieving the A182G point mutation in NRAS exon 3 by site-directed mutagenesis.*

Assay ID	Public RefSeq	Gene Name
Hs00204885_m1	NM_014324	AMACR
Hs00231713_m1	NM_13442	CREB1
Hs00171346	NM_181500	CUTL1
Hs00190723_m1	NM_004675	DIRAS3
Hs00183740_m1	NM_012242	DKK1
Hs99999905_m1	NM_002046	GAPDH
Hs00172878_m1	NM_005524	HES1
Hs00194353_m1	NM_005564	LCN2
Hs00212390_m1	NM_016269	LEF1
Hs00261671_m1	NM_032603	LOXL3
Hs00611472_m1	NM_199072	MDFIC
Hs00231653_m1	NM_003998	NFKB1
Hs00173615_m1	NM_002852	PTX3
Hs00398096_m1	NM_005841	SPRY1
Hs00332674_m1	NM_175866	UHMK1

Table 2.6. *TaqMan* assays-on-demand used for Microarray data validation by *RealTime PCR*.

3. CO-EXPRESSION OF ONCOGENIC NRAS AND BRAF IN MELANOMA CELLS ELICITS NEGATIVE SELECTION BY CELL AUTONOMOUS AND EXTRINSIC MECHANISMS.

3.1 Mutual exclusivity of *NRAS* and *BRAF* oncogenic mutations at the single cell level.

3.1.1 Introduction.

The RAS/RAF/MEK/ERK signalling kinase cascade that transduces extra cellular mitogenic signals to the cell nucleus has been shown to be constitutively activated in human cutaneous melanoma due either to oncogenic mutations in *BRAF* or *NRAS*, or through autocrine growth factor stimulation (Satyamoorthy et al., 2003; Smalley, 2003; Sebolt-Leopold and Herrera, 2004). The *BRAF* gene encodes a serine/threonine kinase, which couples signalling from activated RAS to downstream MAPK kinases (Wellbrock et al., 2004). Mutations in the *BRAF* gene mainly affect exons 11 and 15 encoding the kinase activation domain (Davies et al., 2002; Mercer and Pritchard, 2003; Garnett and Marais, 2004) and are found at high frequency in human melanoma. In more than 90% of melanomas with a mutated *BRAF*, a single-base mutation at nucleotide position 1799 of exon 15 leads to a valine to glutamate (V600E) substitution (Davies et al., 2002; Mercer and Pritchard, 2003; Garnett and Marais, 2004; Gray-Schopfer et al., 2005). The *BRAF*^{V600E} mutation significantly increases kinase activity of BRAF and induces constitutive ERK phosphorylation and focus formation in NIH3T3 cells (Davies et al.,

2002; Garnett and Marais, 2004; Wan et al., 2004). In melanoma, depletion of mutant *BRAF* by RNA interference (RNAi) techniques has been shown to inhibit MAPK cascade, to cause growth arrest, to promote apoptosis and to abrogate transformation (Gray-Schopfer et al., 2005). Although benign nevi also display *BRAF*^{V600E}, recent data suggest that such mutation is more frequent in growing nevi that tend to progress to malignancy (Pollock et al., 2003; Loewe et al., 2004).

Activating mutations in the *NRAS* gene are the other prevalent genetic hallmarks of melanomas, occurring in more than 20% of primary and more than 30% of metastatic melanomas, and mainly in lesions from sunexposed sites (Ball et al., 1994; Jiveskog et al., 1998; Omholt et al., 2003). The most common point mutations affect codon 61 (Q61R and Q61K) and result in the expression of RAS oncoproteins with a single substituted amino acid that remain constitutively GTP bound and active (Malumbres and Barbacid, 2003). Active GTP-bound RAS protein interacts with a wide range of signalling effectors including RAF kinases, phosphatidylinositol 3-kinase (PI3K), Ral-guanine nucleotide exchange factors (Ral-GDS, RGL, RLF and RGL3), NORE/RASSF1 and phospholipase C (Repasky et al., 2004).

In spite of the biological relevance of the RAS/RAF/MEK/ERK signalling cascade and of the high frequency of mutated *BRAF* or *NRAS* in human melanoma, simultaneous presence of *BRAF* and *NRAS* activating mutations in the same tumor are rare (Garnett and Marais, 2004). Moreover, in few exceptional cases in which a *BRAF* mutation was found together with a transforming *NRAS* mutation, the *BRAF* mutation did not include the V600E change (Gorden et al., 2003). Similarly, *NRAS* mutations detected in *BRAF*^{V600E} tumors were rare and not transforming (Brose et al., 2002). These data are consistent with

the hypothesis that “double mutant” tumors, harbouring both activated *BRAF* and *NRAS*, may not occur naturally, since such neoplastic cells may either lack a survival advantage, or even be selected against during tumorigenesis. Unlike melanomas, however, coincident activating mutations in *BRAF* and *NRAS* genes have been found in nevi (Pollock et al., 2003).

To explore the possibility that activating *BRAF* and *NRAS* mutations could coexist even in the same melanoma, we adopted the highly sensitive mutant allele-specific amplification (MASA) PCR technique (Hasegawa et al., 1995; Linard et al., 2002; Miller et al., 2004). By screening a panel of 14 human primary and metastatic melanomas, we found 1 tumor carrying the *BRAF*^{V600E} mutation together with a transforming *NRAS* mutation (Q61R). However, *BRAF* and *NRAS* mutations were mutually exclusive at the single-cell level, as shown by analysis of melanoma clones isolated from this tumor.

3.1.2 Results.

3.1.2.1 Identification of melanomas simultaneously expressing *NRAS* and *BRAF* activating mutations by MASA-PCR.

For this study, 14 short term melanoma cell lines previously characterized for either *NRAS* or *BRAF* activating mutations were chosen (Daniotti et al., 2004; Lupetti et al., 1994). As detected by sequence analysis or oligonucleotide hybridization (Daniotti et al., 2004; Lupetti et al., 1994), 7 out of the 14 tumors harbour the *NRAS*^{Q61R} mutation, 1 tumor the *NRAS*^{G12S} mutation and 6 tumors the *BRAF*^{V600E} mutation, all in heterozygosis (Table 3.1).

In order to assess whether any of these cell lines carried both *NRAS* and *BRAF* mutations, DNA isolated from these tumors was re-analyzed for the presence of both mutations using the sensitive MASA-PCR method (Hasegawa et al., 1995; Linard et al., 2002; Miller et al., 2004). Published forward primers designed with two mismatches at their 3' were used to amplify mutant *NRAS*^{Q61R} and *BRAF*^{V600E}, and control forward primers, matching with the wild type genes, were used to amplify both wild type and mutant sequences (Linard et al., 2002; Xu et al., 2003). After 35-cycle amplification, single bands corresponding to a single mutated allele (*NRAS*^{Q61R} and *BRAF*^{V600E}) were detected with no evidence of double mutated samples. In order to show the reliability of MASA-PCR for genotyping, a representative experiment performed on 5 *BRAF*^{V600E} (i.e. 10538P, 15392M, 4023M, 18732M, 18588M) and 2 *NRAS*^{Q61R} (i.e. 665/2, 8959M) tumors is reported in Figure 3.1 A. Only one mutated allele was detected in each tumor. As a control, all samples could be amplified by control primers which anneal with either *NRAS* exon 3 or *BRAF* exon 15 independently from the mutations (Figure 3.1 A). All *NRAS* mutated samples were re-tested for *BRAF*^{V600E} mutation and all *BRAF*^{V600E} for the most common *NRAS*^{Q61R} mutation by MASA-PCR at higher number of cycles. No *NRAS* mutations were found in *BRAF* mutated samples (data not shown). A *BRAF* amplification signal, representative for the *BRAF* mutation, could however be clearly detected when MASA-PCR amplification was conducted for 45 cycles (Figure 3.1 B). Similarly to standard MASA-PCR (35 cycles), chromatographs of amplified exon 3 *NRAS* and exon 15 *BRAF* fragments could only confirm the A/G mutation (Q61R) for *NRAS* while displaying a *BRAF* wild type sequence (Figure 3.1 C). All other *NRAS*^{Q61R} tumors,

including 2 additional metastases of the same patient (665/1 and 665/R), remained negative for the *BRAF*^{V600E} mutation even when tested at 45 cycles (data not shown).

A titration assay was then performed to compare sensitivity between MASA-PCR and direct fluorescent sequencing (Figure 3.2 A and B). Decreasing proportions of genomic DNA (10-0.1%) from *BRAF*^{V600E} melanoma 18732M were mixed with a fixed amount of genomic DNA from a human lymphoblastoid cell line (LCL) carrying only the wild type *BRAF* alleles. Both direct fluorescent sequencing and MASA-PCR were performed in these samples. Direct fluorescent sequencing required at least 10% of mutant DNA in the total mixture to clearly detect *BRAF*^{V600E} mutation (Figure 3.2 A). Below the 10% threshold, the presence of a mutant spike could not reliably be distinguished from background on the electropherogram (Figure 3.2 A). As described by Miller et al. (2004), standard 35 cycles MASA-PCR detected mutant DNA with higher sensitivity (5%) (Figure 3.2 B, left panel). When the reaction was conducted at 45 cycles, mutated *BRAF*^{V600E} could be detected also when mutant DNA represented only 0.5% of the mixture (Figure 3.2 B, right panel).

Taken together these results suggested that a minor subpopulation of cells in melanoma 665/2 carry *BRAF*^{V600E} at frequency below detection by most standard methods. The presence in the same lesion of *BRAF* and *NRAS* mutated cells was not a feature shared by two additional metastases of the same patient (665/1 and 665/R), since they only displayed the *NRAS*^{Q61R} mutation (data not shown).

3.1.2.2 *NRAS*^{Q61R} and *BRAF*^{V600E} mutations are mutually exclusive at the single cell level.

In order to assess whether *NRAS* and *BRAF*, found mutated in the same lesion, could coexist at the single cell level, we examined, by MASA-PCR as well as by direct sequencing, 21 clones derived by cloning in soft-agar Me 655/2 cells after a few *in vitro* passages (Anichini et al., 1986). The results obtained with both mutational analyses were concordant and indicated that 5 of the 21 clones (2/4, 2/14, 2/17, 2/51, 2/60) displayed the activating Q61R mutation at one of the alleles, whereas all remaining clones had wild type *NRAS* alleles, in agreement with a previous report (Table 3.2 and Figure 3.3 A and B) (Lupetti et al., 1994).

Representative results of both MASA-PCR and direct sequencing are shown in Figure 3.3. Strikingly, the *BRAF*^{V600E} mutation was detected in all the clones with wild type *NRAS*, while clones with mutated *NRAS* were wild type for *BRAF* (Table 3.2 and Figure 3.3 A and B, for representative results).

To rule out the possibility that the *BRAF* mutation could be gained *in vitro*, as an adaptive response to cell culture or to cloning in soft agar, 35 melanoma clones obtained from the two additional *NRAS*^{Q61R} metastatic lesions (665/1 and 665/R) from the same patient, and from a primary *NRAS*^{Q61R} melanoma of a different patient (5810P), were analyzed. All these tumors showed to harbour exclusively *NRAS* mutations (Table 3.2).

3.1.3 Discussion

The results of this study indicate that activating mutations of both *NRAS*^{Q61R} and *BRAF* (hot-spot codon 600) genes can coexist in the same human melanoma. However,

although fluorescent sequencing or standard MASA-PCR (35 cycles) could detect one of the two mutations (*NRAS*^{Q61R}), neither of these techniques, including pyrosequencing (Sivertsson et al., 2002) (data not shown), did reach the necessary sensitivity to detect the presence of the second mutation (*BRAF*^{V600E}) that could be evidenced only through the use of 45 cycles MASA-PCR. The higher sensitivity level required to detect the *BRAF* mutation suggested that this genetic lesion affected a minor subpopulation of cells. Our titration experiments confirmed the increased sensitivity of MASA-PCR, even at 35 cycles, compared to conventional sequencing approaches (Miller et al., 2004).

Such experiments further demonstrated that the specificity of MASA-PCR is maintained at high number of cycles (45 cycles) at which it could reliably detect mutated *BRAF* DNA even when diluted in wild-type DNA at percentages as low as 0.25%, assuming heterozygosis for this allele.

Future studies, by exploiting MASA-PCR as well as novel techniques with even greater sensibility (Lilleberg et al., 2004) in large sets of melanoma patients, are needed to assess the real frequency, at the population level, of tumors that contain mutations in both *NRAS* and *BRAF* oncogenes.

Concomitant activation of *NRAS* and *BRAF* has been detected in microdissected nevi samples (Pollock et al., 2003). However, the microdissection technique did not allow for assessing whether both alterations could coexist at the same cell level. This issue could be tested in this study through the mutational analysis of melanoma clones isolated from 665/2 tumor. Our data demonstrate that *NRAS*^{Q61R} and *BRAF*^{V600E} mutations in 665/2 melanoma are mutually exclusive at the single-cell level providing further support to the hypothesis that expression of the two mutations may not occur in the same

neoplastic cell. Whether concurrent $NRAS^{Q61R}$ and $BRAF^{V600E}$ mutations, if present in the same cell, are incompatible with cell survival or contributes to an irreversible growth arrest remains to be determined.

3.2 Co-expression of oncogenic NRAS and BRAF in the same melanoma cell activates a senescent phenotype and increases susceptibility to cell-mediated cytotoxicity.

3.2.1 Introduction.

Activating *RAS* mutations, mostly at codon 61 of *NRAS*, have been identified in 14% of melanoma lesions arising from soles or palms and in 20% of tumors arising from skin with chronic sun exposure, but in only 3-5% of tumors arising from skin areas with intermittent sun exposure, or from areas protected from the sun (Curtin et al., 2005). In contrast, *BRAF* mutations, mostly at codon 600, have been found in 78% of the tumors from skin with intermittent UV exposure, the most common form of the disease (Curtin et al., 2005). In spite of the overall high frequency of activated *NRAS* or *BRAF* in melanoma lesions, mutations of these two oncogenes have been found to be mutually exclusive in the same tumor (Curtin et al., 2005; Davies et al., 2002; Gray-Schopfer et al., 2005). The lack of association between *KRAS* and *BRAF* mutations has also been found in colon, endometrial and ovarian carcinoma lesions (Rajagopalan et al., 2002; Feng et al., 2005; Singer et al., 2003) and between *K/N/HRAS* and *BRAF* mutations in thyroid carcinoma lesions (Giordano et al., 2005). However, human melanomas harboring both *NRAS* and *BRAF* mutations have been recently described (Kumar et al., 2003; Akslen et al., 2005; Sensi et al., 2006; Goel et al., 2006). Nevertheless, analysis of clones isolated from one of such double mutant tumors indicated that the two activating mutations are mutually exclusive at the single cell level (Sensi et al., 2006).

These findings have suggested an epistatic relationship between *RAS* and *BRAF* oncogenes (Rajagopalan et al., 2002). According to such a model, mutation of both *BRAF*

and *RAS*, in cells where one or the other of these oncogenes is already activated, may not provide an additional significant growth/survival advantage. However, an alternative mechanism for mutually exclusive *NRAS* and *BRAF* mutations in melanoma cells is represented by “synthetic lethality”. As recently reviewed by Kaelin, two genes are said to be synthetically lethal when mutations in either one is compatible with cell survival, while simultaneous mutations in both genes is not (Kaelin et al., 2005). Although synthetic lethality usually involves loss-of-function mutations, it could also result from gain-of-function changes, as in the instance of the activating *NRAS* and *BRAF* mutations found in melanoma cells.

To provide insights into the mechanisms that may explain mutual exclusion of activated *NRAS* and *BRAF* at the single cell level, we developed a constitutive and a doxycycline-regulated system for expressing an oncogenic *NRAS* (*NRAS*^{Q61R}) in a metastatic melanoma clone (665/2/21) harboring wild type *RAS* alleles (*HRAS*, *KRAS* and *NRAS*) and a 1798 T/A *BRAF* mutation (*BRAF*^{V600E}) at one of the *BRAF* alleles. The results to be presented indicate that co-expression of activated *NRAS* and *BRAF* in the same melanoma cell activates a senescence program and boosts melanoma cell recognition by cytotoxic lymphocytes.

3.2.2 Results

3.2.2.1 Constitutive or doxycycline-regulated expression of mutated *NRAS*^{Q61R} in a *BRAF*^{V600E} melanoma clone.

Melanoma clone 2/21 expresses wild type *HRAS*, *KRAS*, *NRAS* genes (Anichini et al., 1989), but harbors a heterozygous *BRAF*^{V600E} mutation (Figure 3.4) and bears a homozygous inactivating Tyr->His mutation at codon 236 in exon 7 of *p53* gene (Anichini et al., 1999) and a homozygous deletion of exon 3 of the *CDKN2A* locus that prevents p16/*INK4A* and p14/*ARF* expression (Figure 3.4). This clone was used for generating cells with constitutive or inducible expression of an exogenous *NRAS*^{Q61R}. Constitutive *NRAS*^{Q61R} expression was achieved by transfecting with pcDNA3.1-Flag*NRAS*^{Q61R}. Out of 100 clones initially isolated, 54 grew in selective medium and 18 of the latter maintained the mutated insert. However, only 1 out of the 18 (thereafter named 21*NRAS*^{Q61R}) expressed the exogenous mutated *NRAS* at the protein level (Figure 3.5 A). The mutated *NRAS* could be distinguished from the endogenous *NRAS* thanks to the reduced electrophoretic mobility caused by the addition of the Flag peptide to the *NRAS* transgene (Figure 3.6 A). To generate cells with inducible *NRAS*^{Q61R} expression, clone 21 cells were initially transfected with the regulatory pTet-Off vector. Clones expressing a functional regulator tTA protein were identified by a luciferase assay detecting Dox-regulated light emission 48 hours after transient transfection with the pTRE2hyg-*Luc* reporter plasmid. One of the 22 responsive clones, named 21Off, was selected for subsequent experiments, since it showed Dox dose-dependent RLU reduction, and at 1 µg/mL Dox reached RLU values similar to those displayed by non

transfected cells (Figure 3.6 B). 21Off cells were then transfected with plasmids encoding mutated (pTRE2hyg-Flag*NRAS*^{Q61R}) or wild type (pTRE2hyg-Flag*NRAS*^{WT}) *NRAS*. Out of 48 hygromycin-resistant clones, transfected with pTRE2hyg-Flag*NRAS*^{Q61R}, 8 clones grew in selective medium; however, only 2 of them expressed the oncogenic *NRAS* (Figure 3.5 B). The presence of mutated *NRAS* and *BRAF* in these two clones was confirmed by sequence analysis (Figure 3.4 B). In contrast, 30 out of 48 clones transfected with pTRE2hyg-Flag*NRAS*^{WT} kept growing and expressed the wild-type inserted *NRAS* (Figure 3.5 B). Expression and Dox-dependent regulation of the transgenic mutant or wild type *NRAS* was evaluated by Western blotting analysis (Figure 3.6 A). In the absence of Dox, oncogenic *NRAS* as well as the wild type endogenous *NRAS*, were detected using *NRAS*-specific mAb. Addition of Dox (1 µg/mL) to the culture medium inactivated the transcription of the exogenous *NRAS* in a time-dependent fashion. The gene was completely silenced following a 48 hours incubation (Figure 3.6 C). In contrast, no effect was detected on the transcription of the endogenous gene. A clone with inducible *NRAS*^{Q61R} expression (thereafter named 21*NRAS*^{61ON} or 21*NRAS*^{61OFF} depending on expression or not of the exogenous mutant *NRAS*) and a clone with inducible wild type *NRAS* expression (thereafter named 21*NRAS*^{WTON} or 21*NRAS*^{WTOFF} depending on expression or not of the exogenous wt *NRAS*) were selected for further experiments.

3.2.2.2 NRAS activation, ERK and Akt phosphorylation in “double mutant” 21NRAS^{61ON} cells.

To assess the function of the exogenous activated *NRAS* oncogene, we used the RAS-binding domain of BRAF as a RAS-GTP-dependent probe (de Roij et al., 1997) to immunoprecipitate active NRAS from cell lysates. As shown in Figure 3.7, more activated NRAS protein was immunoprecipitated from 21NRAS^{61ON} than from 21NRAS^{61OFF} cells. Dox-regulated expression of the mutated NRAS^{Q61R} led to levels of active NRAS protein similar to those found in the human melanoma cell line 4473M harboring a mutated *NRAS*^{Q61R} allele (Figure 3.7). In addition, active NRAS levels were similar in 21NRAS^{61OFF} and in the parental 2/21 cells (Figure 3.7). Since NRAS mutations can result in dual activation of MAPK and PI3K pathways (Curtin et al., 1995; Goel et al., 2006), we then evaluated levels of total and phosphorylated ERK and AKT in 2/21 cells with expressed or silenced NRAS^{Q61R}. Total endogenous NRAS and total amount of ERK proteins were similar in all cell lines (Figure 3.7). In contrast, levels of phosphorylated ERK were much higher in 21NRAS^{61ON} cells compared not only to 21NRAS^{61OFF} cells, but even to the parental clone 2/21 expressing only BRAF^{V600E}, and to melanoma 4473M expressing only NRAS^{Q61R}. Moreover, level of total AKT was similar in all samples, while phosphorylated AKT was found only in cells bearing an activated in NRAS^{Q61R} (21NRAS^{61ON} cells and 4473M sample). PTEN protein expression was found in all the samples (Figure 3.7). Similar results were obtained by comparing the constitutive 21NRAS^{Q61R} transfectant with the parental clone 2/21 cells (data not shown). These results indicate that co-expression of activated *NRAS* and *BRAF* oncogenes in the

same melanoma cells is associated with over-activation of components of the RAS-effector cascade.

3.2.2.3 A senescent phenotype develops in $BRAF^{V600E}$ melanoma cells after constitutive or inducible expression of $NRAS^{Q61R}$.

In vitro growth properties of the 21NRAS^{Q61R} constitutive transfectant were similar to those of the parental clone 2/21 in the early *in vitro* passages. However, after the 12th *in vitro* passage, 21NRAS^{Q61R} cells began to lengthen their doubling time (data not shown). Cell cycle analysis between the 12th and the 17th *in vitro* passage indicated a progressive accumulation of 21NRAS^{Q61R} cells in the G₀/G₁ phase of the cell cycle (from 25.75% to 51.90%, Figure 3.8 A) and a corresponding decrease in the fraction of cells in S phase (from 64.12% to 29.38%). No such changes were seen either in the parental clone 2/21 or in a clone that underwent the same selection procedure as 21NRAS^{Q61R} but did not express mutated NRAS (21NRAS^{WT} in Figure 3.8 A). Furthermore, by a colorimetric assay, using X-gal as a substrate at pH 6.0, 21NRAS^{Q61R} cells at the 12th *in vitro* passage began to stain positive for the senescence-associated marker SA-β-Gal (Figure 3.8 B), consistent with activation of cellular senescence (Dimri, 1995), while both 21NRAS^{WT} and clone 2/21 cells remained negative.

Growth of 21NRAS^{61ON} cells, in comparison to 21NRAS^{61OFF}, also slowed down after the 10th *in vitro* passage. Proliferation was therefore quantified using an MTS assay. As shown in Figure 3.9 A, OD_{490nm} values were similar in 2/21 cells cultured in presence or absence of Dox, in 21NRAS^{WTON} and 21NRAS^{WTOFF} (at different *in vitro* passages), in 21NRAS^{61ON} (9th *in vitro* passage) and 21NRAS^{61OFF} (both at 9th and 15th *in vitro*

passages). In contrast, OD values were significantly lower in 21NRAS^{61ON} cells, at the 15th *in vitro* passage, compared to all other samples. A reduced growth of 21NRAS^{61ON} cells at the 15th *in vitro* passage was also observed following one week culture in Matrigel (Figure 3.9 B, upper panel), an ECM protein gel produced by the EHS tumor and known to be highly effective in supporting cell proliferation (Albini, 1998; Fridman et al., 1991). After seeding into Matrigel, cells expressing NRAS^{Q61R}, either did not divide or formed much smaller colonies than 21NRAS^{61OFF} cells (Figure 3.9 B, upper panel), while 21NRAS^{WTON} and 21NRAS^{WTOFF} could grow to a similar extent (Figure 3.9 B, upper panel). In addition, beyond the 10th *in vitro* passage, 21NRAS^{61ON} cells became larger and flatter than 21NRAS^{61OFF} cells (Figure 3.9 B, middle panel), a cell morphology usually associated with a senescent phenotype (Campisi 2005; Shay et al., 2004), and not observed in either 21NRAS^{WTON} or 21NRAS^{WTOFF} (Figure 3.9 B, middle panel). At this stage, 21NRAS^{61ON} cells, like the constitutive 21NRAS^{Q61R}, began to stain positive for the senescence-associated marker SA-β-Gal activity, while 21NRAS^{61OFF}, as well as 21NRAS^{WTON} and 21NRAS^{WTOFF} did not (Figure 3.9 B, lower panel). 21NRAS^{61ON} and 21NRAS^{61OFF} cells were then evaluated for the expression of additional markers of senescence. Phosphorylated AMP-activated protein kinase (pAMPK), a serine/threonine protein kinase recently shown to be upregulated in senescent cells (Hardie 2005; Xiang et al., 2004; Wang et al., 2003), was detected in the inducible 21NRAS^{61ON} and in the constitutive 21NRAS^{Q61R} transfectants, but only at late *in vitro* passages (15th and 14th, respectively, Figure 3.10), while it was not expressed in all other cells including pre-senescent 21NRAS^{61ON} (9th *in vitro* passage) and 21NRAS^{Q61R} (8th *in vitro* passage). Cyclin-dependent kinase inhibitor p21^{waf1/Cip1} (p21), a mediator of growth arrest (Chang

et al., 2000), could not be detected in non-senescent 21NRAS^{61OFF}, 21NRAS^{WTON} and 21NRAS^{WTOFF} (all passages tested), but was expressed in 21NRAS^{61ON} and 21NRAS^{Q61R}, at the senescent and the pre-senescent stages. Both p21 and pAMPK were not detected in melanoma cells expressing only BRAF^{V600E} (clone 2/21), or only NRAS^{Q61R} (4473M). Expression of several molecules involved in p53 and p16/pRB pathways, like p53 itself, PCNA, MDM2, phosphorylated and underphosphorylated RB did not change when activated NRAS and BRAF oncogenes were expressed both in the same cell (data not shown). Taken together, these results suggest that co-expression of activated NRAS and BRAF in the same melanoma cells can activate a senescence program that induces cell growth arrest.

3.2.2.4 Co-expression of activated NRAS and BRAF impacts on immune recognition and antigen processing machinery phenotype of melanoma cells.

Additional mechanisms may contribute to selection against “double mutant” tumor cells bearing activated *NRAS* and activated *BRAF* oncogenes. To provide evidence in support of this possibility, we evaluated susceptibility of constitutive and inducible NRAS^{Q61R} transfectants to cell mediated cytotoxicity, as the immune system is the main extrinsic suppressor of tumor growth (Dunn et al., 2002). The constitutive transfectant 21NRAS^{Q61R} was significantly ($p=0.0078$) more susceptible, in terms of lytic units, to cell-mediated lysis, by IL-2 activated non-specific LAK effectors generated from six different donors, compared to parental clone 2/21 cells (Figure 3.11 A). A limiting dilution assay for the determination of cytotoxic T cell effector frequency (Mortarini et al., 2000) was then used to compare 21NRAS^{61ON} and 21NRAS^{61OFF} cells for HLA-

restricted and non-restricted cytotoxic T cell-mediated lysis. To this end, an alloreactive T cell line, established from PBL of an HLA-mismatched donor by repeated MLTC stimulation with 21NRAS^{61OFF}, was used. Frequency of all lytic effectors (i.e. independently from inhibition of lysis by anti-HLA-class I mAb) and of HLA-class I-restricted alloreactive cytotoxic T cell effectors cells was much higher on 21NRAS^{61ON} than on 21NRAS^{61OFF} targets, in spite of the fact that the effectors had been selected on the latter targets (Figure 3.11 B). Lysis of 21NRAS^{61ON} was significantly higher than lysis of 21NRAS^{61OFF} ($p=0.0189$) (Figure 3.11 C) even by the autologous HLA class I-restricted CTL clone 8B3 that was previously isolated from tumor-infiltrating lymphocytes of patient 665 and was shown to be able to specifically lyse melanoma 665/2 cells (Anichini et al., 1989).

To determine whether the increased susceptibility to cell mediated lysis of 21NRAS^{61ON} cells reflected changes in the expression of molecules involved in target-cytotoxic cell interactions, 21NRAS^{61ON} and 21NRAS^{61OFF} cells were then compared for the expression of a large number of cell surface and intra-cellular molecules, including HLA class I antigen processing machinery (APM) components. These molecules play a major role in the generation of HLA class I-peptide complexes which are recognized by CD8⁺ CTL. Flow cytometry analysis of cells intracellularly stained with APM component-specific mAb showed a higher expression of constitutive proteasome subunits delta and MB1, of immunoproteasome subunits LMP2, LMP7 and LMP10 and of chaperones calnexin and calreticulin and ERp57 in 21NRAS^{61ON} cells than in 21NRAS^{61OFF} cells (Figure 3.12 A). No differences were detected between the two cell types in the expression of the transporter associated with antigen processing (TAP)

subunits TAP-1 and TAP-2 and of the chaperone tapasin. Furthermore, 21NRAS^{61ON} cells and 21NRAS^{61OFF} cells did not differ in the cell surface expression of HLA-class I antigens, HLA-DR, -DQ, -DP antigens, α v β 3 integrin β 1, β 4, α 2, α 3, α 4, α 5, and α 6 integrin subunits, and adhesion molecules ICAM-1 and LFA-3 (Figure 3.12 A). In addition, all APM molecules found differentially expressed in 21NRAS^{61ON} cells compared to 21NRAS^{61OFF} cells showed a similar expression pattern in 21Off cells treated or not with Dox (Figure 3.12 B, upper panel). Moreover, 2/21 cells transfected with an inducible wild type NRAS (21NRAS^{WT}) did not show differential expression of the APM components when treated or not with Dox (Figure 3.12 B, lower panel). Lastly, the death receptors Fas/CD95, TRAIL-R1 TRAIL-R2, TRAIL-R3 and TRAIL-R4 were not differentially expressed in 21NRAS^{61ON} and 21NRAS^{61OFF} cells (data not shown).

Taken together, these data indicate that co-expression of activating NRAS and BRAF mutations in the same melanoma cells may impact on recognition by both non-specific and HLA-restricted cytotoxic effectors and on expression of several APM molecules involved in the generation of HLA-class I-peptide complexes recognized by T cells.

3.2.3 Discussion

Early evidence on mutual exclusion of activating *KRAS* and *BRAF* mutations in colorectal cancer suggested that *RAS* and *BRAF* oncogenes may exert equivalent effects in tumorigenesis, due to the common MEK-ERK effector cascade they regulate (Rajagopalan et al., 2002). In agreement with this possibility, concomitant mutation of *BRAF* and loss of *PTEN* have been described in melanoma (Goel et al., 2006; Tso et al.,

2000). Such condition could induce biochemical effects similar to an *NRAS* mutation, as activated *NRAS* can result in dual activation of MAPK and PI3K pathways (Curtin et al., 2005; Goel et al., 2006). In agreement, in this study, both these pathways were activated in melanoma clone 2/21 after expression of a mutated *NRAS* oncogene. However, other studies have identified *NRAS* or *BRAF* mutation-specific gene expression profiles in thyroid carcinoma and in melanoma (Giordano et al., 2005; Pavey et al., 2005), and a selective susceptibility to MEK inhibition in *BRAF* mutant tumors, but not in *NRAS* mutant tumors (Solit et al., 2006). These data suggest that activated *NRAS* and *BRAF* oncogenes may not necessarily drive equivalent phenotypic effects. In addition, analysis of clones isolated from a “double mutant” melanoma indicated that *NRAS* and *BRAF* activating mutations do not coexist in the same cells (Sensi et al., 2006), suggesting that the two mutations may be incompatible with cell survival. This possibility is in agreement with the concept of synthetic lethality recently reviewed by Kaelin (Kaelin, 2005). Genes having a synthetic lethal relationship may be identified among those that behave as “double-edged” swords for neoplastic cells, as able to deliver signals that may promote or inhibit cell proliferation and/or survival (Kaelin, 2005). Interestingly, both activated RAS and RAF proteins can play such contrasting roles depending on cell context and on the function/deficiency of other pathways including PI3K, RB and the p53 pathways. Thus, activated *NRAS* contributes to neoplastic transformation of human melanocytes and to development of invasive melanomas, when the RB and p53 pathways are inhibited (Chudnovsky et al., 2005). Similarly, activated *BRAF* is a transforming oncogene in immortalized melanocytes (Wellbrock et al., 2004). On the other hand, several recent papers have shown that pre-malignant cells from different tissues can

activate a senescence program in response to oncogenic RAS (Shay et al., 2004), and that activated BRAF triggers senescence in melanocytes from nevi (Michaloglou et al., 2005).

In agreement with the hypothesis that oncogenic NRAS and BRAF may be synthetically lethal, this study shows that, after the first 9-10 *in vitro* passages, the constitutive and the Dox-regulated transfectants expressing both NRAS^{Q61R} and BRAF^{V600E} had reduced *in vitro* growth compared to control cells, progressive accumulation in G₀/G₁ phase of the cell cycle and positive staining for the senescence-associated acidic β -galactosidase (SA- β -Gal). Furthermore, analysis of the Dox-regulated transfectants provided additional evidence for senescence, as indicated by increased levels of pAMPK and of the cyclin-dependent kinase inhibitor p21. Activation of apoptosis, as an alternative mechanism leading to elimination of the cells expressing NRAS^{Q61R} and BRAF^{V600E} cannot be ruled out. However, no evidence consistent with programmed cell death, such as cell shrinkage, membrane blebbing and formation of apoptotic bodies (Kerr et al., 1972), was observed in the cultures. Taken together, these findings suggest that constitutive or conditional expression of a mutant NRAS oncogene in a metastatic melanoma naturally expressing a BRAF^{V600E} oncogene is sufficient, in the absence cell cycle regulators, like p16/pRB and p14^{ARF}/p53 (Fridman et al., 1991; Campisi, 2005), to activate senescence.

Cellular senescence is a genetic program leading to irreversible cell cycle arrest and can be mediated by several genes, including some oncogenes (Campisi, 2005; Shay et al., 2004). In fact, in primary murine and human cells activated components of the RAS-RAF-MAPK signaling cascade are known to trigger senescence responses that prevent the unlimited expansion of these cell types (Serrano et al., 1995; Sewing et al.,

1997; Lin et al., 1998). The selective association of senescence in 21NRAS^{61ON} cells with p21 up-regulation is in agreement with the described requirement for this cell cycle inhibitor in senescence associated with high levels of oncogenic RAS activity (Chang et al., 2000; Lin et al., 1998). Although p21 is the main target of p53, several p53-independent mechanisms for its induction have also been reported. They may underlie p21 induction in double mutant 21NRAS^{61ON} cells, where p53 is functionally deficient (data not shown). For example, in the p53-negative U937 myeloid cell line, NRAS-induced senescence was dependent on p21 upregulation mediated by interferon regulatory factor-1 (Passioura et al., 2005). In human diploid fibroblasts undergoing replicative senescence, enzymatic components of the cGMP pathway were inhibited and exposure of colorectal cancer cells to a pharmacological inhibitor of cGMP synthesis induced activation of p21 and senescence (Lodygin et al., 2002). In melanoma cells and melanocytes, senescence appears to be regulated by the basic helix loop-helix leucine zipper microphthalmia-associated transcription factor (MITF) (Steingrimsson et al., 2004). MITF is able to induce a G₁ cell cycle arrest that is dependent on p21 activation through modulation of its direct transcriptional repressor Tbx2 (Carreira et al., 2005; Vance et al., 2005). BRAF^{V600E} signaling appears to down-regulate MITF at a level compatible with cell proliferation, since high MITF levels have anti-proliferative effects (Wellbrock et al., 2005). Low MITF levels are linked to reduced survival rates and increased metastases in patients with intermediate thickness melanoma (Salti et al., 2000). However, induction of p21 in 21NRAS^{61ON} cells is independent from MITF up-regulation, since both senescent 21NRAS^{61ON} and non-senescent 21NRAS^{61OFF} cells had similar levels of MITF protein expression (data not shown). It cannot however be ruled

out that differential MITF transcriptional activity as a result of increased MAP kinase signaling could play some role in senescence induction (Widlund et al., 2003). In addition to the induction of p21 expression, senescent 21NRAS^{61ON} cells displayed up-regulation of AMPK. This metabolic enzyme is a key regulator of response to low ATP levels and is induced to prevent ATP-consuming processes under conditions of low cellular energy (Hardie, 2005). Recently, AMPK activity was found to be increased in cells undergoing senescence, while its over-activation promoted senescence in primary human fibroblasts (Wang et al., 2003; Xiang et al., 2004). Role of pAMPK induction in melanoma cells needs to be further explored and will be deeper investigated in Chapter 4 of this thesis.

Activation of senescence, by co-expression of oncogenic NRAS and BRAF in the same melanoma cells, may not be the only mechanism leading to selection against “double mutant” cells. Since the immune system is the main extrinsic tumor suppressor factor, we evaluated whether cells with expressed or silenced NRAS^{Q61R} had significantly different susceptibility to cell-mediated cytotoxicity by measuring frequencies of both HLA-class I-restricted and non-restricted cytotoxic effectors directed against 21NRAS^{61ON} or 21NRAS^{61OFF}. Due to the lack of autologous lymphocytes, these effectors were generated by repeated restimulation of lymphocytes from an HLA-mismatched donor with 21NRAS^{61OFF} cells in MLTC. The results indicated that the frequency of cytotoxic effectors was markedly higher on 21NRAS^{61ON} than on 21NRAS^{61OFF} either when the cytotoxicity assay was performed in presence or absence of w6/32 mAb to inhibit the HLA class I antigen restricted component of the lysis. Differential lysis of 21NRAS^{61ON} and 21NRAS^{61OFF} cells was observed even by using an autologous HLA class I-restricted CTL clone isolated from TILs and previously characterized (Anichini et

al., 1993). Interestingly, increased lysability of 21NRAS^{61ON} was associated with up-regulation of a specific set of components of the HLA antigen processing machinery that contribute to generation of MHC-peptide complexes recognized by T cells (Lehner et al., 2004).

These data, together with those indicating activation of senescence, are consistent with the possibility that double mutant cells, harboring both activated *NRAS* and *BRAF* oncogenes, may be selected against by cell autonomous and extrinsic mechanisms.

3.3. Identification of proliferation arrest mediators by microarray analysis in melanoma cells.

3.3.1 Introduction.

3.3.1.1 MicroArray technology in cancer research.

Over the past decade, the completion of human genome sequence opened the way to new approaches for gene analysis. Among these novel technologies, microarray represents an efficient approach to extract data of biomedical relevance for a wide range of applications, enabling the study of genome-wide expression patterns in complex biological systems. For these reasons, microarray technology has emerged as an important tool for the characterization of cancer cells (Sears et al., 2007).

In cancer research, this technology has several applications. Indeed, microarrays can provide useful insights into differences in an individual's tumor as compared with constitutional DNA, mRNA and protein expression; across different individuals, comparisons can provide tissue-specific disease signatures with important diagnostic implications. In this way, clusters of genes which are regulated together can be identified. The expression pattern of gene clusters can then be correlated with morphology, clinical behavior or treatment response. This results in the identification of tumor- associated and tumor-specific biomarkers which may help in cancer etiology, diagnosis and therapy investigation (Liotta et al., 2000). Resulting signature profiles can provide a more accurate prognosis and prediction of response to therapy.

Carcinogenesis is a multistep process that is the outcome of the accumulation of several genetic and epigenetic events. In this scenario, DNA Microarray is a promising approach that allows both qualitative and quantitative screening for sequence variations in the genomic DNA of cancer cells. Detection of mutations in cancer is of major importance for both basic understanding of the disease process and clinical practice. DNA array is a powerful and effective tool for detecting specific mutations, small insertions and deletions in non-repetitive sequences. Several studies showed that microarrays are more sensitive, more accurate and faster than classical sequencing approaches (Wen et al., 2000).

On the other hand, cancer phenotype is also partially determined by its transcriptome. Global transcript analysis could have powerful applications in cancer diagnosis and patient management. Microarray expression comparison allows for the obtainment of a panel of up- or down-regulated genes that reveals a relevant molecular fingerprinting of the cellular state and provides a large body of candidate molecular markers of the disease. Combined with knowledge of the clinical importance of disease process, global transcript analysis has powerful applications in cancer diagnosis and patient management.

In addition, gene expression study in pharmacogenomics offers a new way to monitor qualitative and quantitative aspects of treatment efficacy, resistance mechanisms and generation of novel targets (Debouk et al., 1999).

3.3.1.2 Lipocalin 2.

The lipocalin protein family is a large group of small extracellular proteins. Members of this protein family are typically small secreted proteins which are characterized by a range of different molecular-recognition properties. Lipocalins can bind small hydrophobic molecules, such as retinol, or specific cell-surface receptors with which can form macromolecular complexes. This group of proteins exhibit great structural and functional diversity, both within and between species. In the past they have been classified primarily as transport proteins. However, it is now clear that members of the lipocalin family fulfill a variety of different functions. These include roles in retinol transport, cryptic coloration, olfaction, pheromone transport, and the enzymic synthesis of prostaglandins. The lipocalins have also been implicated in the regulation of the immune response and the mediation of cell homeostasis (for a Review: Flower, 1996).

Lipocalin 2 is a member of the lipocalin superfamily and is expressed in granulocytic precursors as well as in numerous epithelial cell types (Friedl et al., 1999; Nielasen et al., 1996). Increased expression of lipocalin 2 accompanies numerous transformations (induction by polyoma, SV40, phorbol ester, and the *neu* oncogene) and human carcinomas (colorectal, hepatic, pancreatic and breast carcinomas), but the action of the protein is not fully understood (for a review Bratt, 2000).

Recently it has been shown that lipocalin 2 can alter the invasive and metastatic behavior of RAS-transformed breast cancer cells *in vitro* and *in vivo* by reversing the Epithelial-Mesenchymal-Transition (EMT)-inducing activity of RAS (Hanai et al., 2005). Numerous pathways have been defined downstream of RAS activation (Downward, 2003). Moreover it has been demonstrated that lipocalin 2 can restore E-cadherin

expression and regulates RAS signaling. The action of lipocalin 2 on the RAS-MAPK signaling pathway presumably lies between RAS and RAF activation (Hanai et al., 2005). In addition, lipocalin 2 can suppress the RAS-induced expression of VEGF in breast cancer cells via down-regulation of MAPK- and PI3K- signaling (Venkatesha et al., 2006). In addition, the expression of the antiangiogenic molecule thrombospondin-1 was found increased in tumors formed by 4T1-ras cells into which lipocalin 2 was stably introduced.

Tumor angiogenesis, assessed via an intradermal tumor angiogenesis assay, was also suppressed by lipocalin 2.

3.3.1.3 LOXL3.

Lysyl oxidase (LOX), a copper-containing amine oxidase, belongs to a heterogeneous family of enzymes that oxidize primary amine substrates to reactive aldehydes. Traditionally, the well known function assigned to LOX proteins is the extracellular catalysis of lysine-derived cross-links in fibrillar collagens and elastin (Csizar, 2001). More recently, diverse roles have been attributed to lysyl oxidase and these novel activities cover a spectrum of diverse biological functions such as developmental regulation, tumor suppression, cell motility, and cellular senescence (Lazarus et al., 1994, Li et al., 2000; Smith-Mungo et al., 1997). Lysyl oxidase has also been shown to have both intracellular and intranuclear locations (Li et al., 1997). Recent studies identified an up-regulation in lysyl oxidase (LOX) expression, in a highly invasive/metastatic human breast cancer cell line, compared with a poorly invasive/nonmetastatic breast cancer cell line (Kirschmann et al., 2002). Moreover, the

correlation of LOX up-regulation and invasive/metastatic potential was additionally demonstrated in rat prostatic tumor cell lines, and human cutaneous and uveal melanoma cell lines. These results provide substantial new evidence that LOX is involved in cancer cell invasion.

Recently, 4 novel members of this amine oxidase family, LOX-like (LOXL), LOXL2, LOXL4 and LOXL3, were identified (Molnar et al., 2003). The LOXL3 protein contains four scavenger receptor cysteine-rich domains in the N terminus in addition to the C-terminal characteristic domains of the LOX family, such as a copper binding domain, a cytokine receptor-like domain and residues for the lysyl-tyrosyl quinone cofactor.

So far, very little is known about the specific cellular biological functions of LOXL3.

3.3.2 Results.

3.3.2.1 NRAS^{Q61R}/BRAF^{V600E} double mutant cell differentially expressed molecules as evaluated by MicroArray profiling.

In order to delineate the molecular mechanism and identify possible mediators of the senescent program induced in melanoma cells co-expressing oncogenic NRAS and BRAF, the highly sensitive CodeLink Human Whole Genome Bioarrays were used for Microarray analysis. RNA extracted from 21NRAS^{61ON} and 21NRAS^{61OFF} cells (for

details about cell lines see chapter 3.2 and Petti et al., 2006) was processed for this analysis. The experiment was carried out with 2 technical replicates for each sample.

Among the 55000 analyzed probes, 321 genes resulted differentially expressed, where 190 were up- and 131 down-regulated (Table 3.3 and Figure 3.13 A). Significantly, modulated genes could be classified in different signaling pathways such as cell proliferation, cell cycle, cell death, response to stress, response to wound and immune response (Figure 3.13 B). A number of modulated genes were then selected for the validation of Microarray data. To this end, the up-regulated CREB1, DKK1, HIC1, HES1, LCN2, LEF1, LOXL3, NFKB1, PTX3, SPRY1, UHMK1 and the down-regulated AMACR, ARHI and CUTL1 were amplified using TaqMan probes (Figure 3.14 A). Expression modulation of the mRNA levels for the 14 targets evaluated by RealTime PCR was similar to fold change values previously obtained from Microarray results (Figure 3.14 B), thus confirming the validation of Microarray experiment.

As a further validation of Microarray data, the protein levels of LOXL3 were then tested by Western Blot. 21NRAS^{61ON} showed higher expression levels of LOXL3 protein than 21NRAS^{61OFF} (Figure 3.14 C). In agreement with the Microarray results, neither 21NRAS^{61OFF} nor parental 2/21 cells expressed high levels of LOXL3 protein (Figure 3.14 C).

3.3.2.2 Lipocalin 2 and LOXL3 are differentially expressed in a panel of melanoma cells.

In order to understand the physiological role of the differentially expressed molecules validated by RealTime PCR and to correlate their expression with melanoma

behavior, such differentially expressed genes as LOXL3 and lipocalin 2, potentially involved in cell proliferation, were chosen for further investigations.

Expression levels of both LOXL3 and lipocalin 2 genes were evaluated in a panel of melanoma samples at the mRNA level (Figure 3.15 A). As evaluated by RealTime experiments, 3 out of 10 melanoma cell lines (8959M, 14362M, 2211M1) expressed LOXL3 at higher levels (Figure 3.15 A). Similar results were obtained for LCN2, where 2 out of 10 melanoma cell lines (15392M and 14362M) showed higher levels of mRNA expression. In addition, protein levels of lipocalin 2 were evaluated by FACS analysis for 1 melanoma which showed higher and 1 melanoma lower expression at the mRNA level. Lipocalin 2 higher expression in 14362M sample was confirmed in comparison to 15392M which resulted negative at the protein level (Figure 3.15 B).

3.3.2.3 Lipocalin 2 protein expression can be silenced in melanoma cells using specific siRNA.

In order to correlate its expression with tumor behavior, modulation of lipocalin 2 protein levels was achieved in melanoma cells. Lipocalin 2 was silenced at the transcriptional level in 14362M which displayed higher basal protein levels compared to the other melanoma cell lines. Silencing of lipocalin 2 using specific siRNA was not yet effective 24 hours after transfection, while it was evident after 48 hours. After 72 hours from transfection, protein levels reached again the basal values, as tested by flow-cytometry analysis (Figure 3.16). Control scrambled siRNA directed against lipocalin 2 scorrelated target, did not follow the same expression modulation (data not shown), thus

indicating that silencing achieved with the siRNA against lipocalin 2 is specific for and only for lipocalin 2 protein.

The induced down-regulation of lipocalin 2 protein will be used for further functional experiments which will clarify lipocalin 2 involvement in melanoma proliferation.

3.4 Discussion

In this section of the thesis, preliminary data obtained to date are shown. However, deep further investigation is needed in order to achieve strong evidences of the studied phenomenon and to obtain more consistent results.

In the first part of this study, expression profile of NRAS^{Q61R}/BRAF^{V600E} double mutant cells was evaluated by using Microarray technology. Data analysis showed that a number of genes implicated in cell proliferation, cell cycle, cell death, response to stress, response to wound and immune response are affected by the concomitant expression of the two oncogenes. Among these genes, interesting molecules which might explain the previously shown senescent mechanism are included. This kind of molecules might play a relevant role in the control of cell proliferation not only in the specific presented cell model, but also in general in melanoma cells. For this reason, the impact of their expression on cell phenotype needs to be assessed in a wide panel of melanoma samples.

Both lipocalin 2 and LOXL3 may play a role in the control of cancer cell proliferation. Among the functions so far reported in literature for lipocalin 2, this molecule has been associated to the reversion of the EMT phenotype (Hanai et al., 2005).

The knowledge so far collected about this molecule suggests that lipocalin 2 might be relevant for a tumor suppression function.

About LOXL3 less information are available from literature. Some reports correlated lysyl-oxydase proteins with senescence phenotype (Smith-Mungo et al., 1997), thus suggesting that also LOXL3 could be involved in a similar mechanism also in tumor cells. Expression of the *LOX* gene was found to inhibit the transforming activity of the *ras* oncogene in NIH 3T3 fibroblasts and was hence named the *ras* recision gene (Contente et al., 1990; Kenyon et al., 1991). Reduced LOX levels were observed in many cancers and derived cell lines (Kaneda et al., 2004), whereas in spontaneous revertants or upon induced phenotypic reversion, higher normal levels of LOX were again seen (Hajnal et al., 1993). These notions suggest that also the LOX related LOXL3 molecule can exploit similar functions which might drive a proliferation arrest mechanism.

Preliminary experiments performed for lipocalin 2 and LOXL3 in a panel of melanoma cell lines were focused on the assessment of their potential role in proliferation arrest in melanoma. As revealed by microarray results obtained from the double mutant model system, both lipocalin 2 and LOXL3 are generally expressed at low levels in most melanomas. It is conceivable that most melanomas express these two molecules at low levels in order to achieve a growth advantage for tumor expansion. Therefore, it can be speculated that lipocalin 2 and LOXL3 might be involved in the promotion of proliferation arrest in melanoma cells thus acquiring a tumor suppressive function. However, this issue needs to be explored and confirmed with future work.

To this end, for a further characterization of the role of lipocalin 2 and LOXL3 in the control of cell proliferation, both inhibition and over- expression of these proteins should

be achieved in melanoma cells. So far, preliminary experiments aimed at the setting of the silencing of lipocalin 2 through the use of siRNA were performed. In one melanoma cell line, selected for the higher basal expression levels of lipocalin 2 at both mRNA and protein level, inhibition of lipocalin 2 protein expression was reached after 48 hours from transfection with specific siRNA. Biological consequences of the induced silencing have to be evaluated in melanoma cells. In order to explore this issue, cell proliferation and cell cycle modulation will be tested. In addition, enhanced expression of lipocalin 2 protein will be induced by using specific constructs and the correspondent phenotype will be then investigated.

Similar approaches will be used also for further explorations about the role of LOXL3 protein in melanoma behavior.

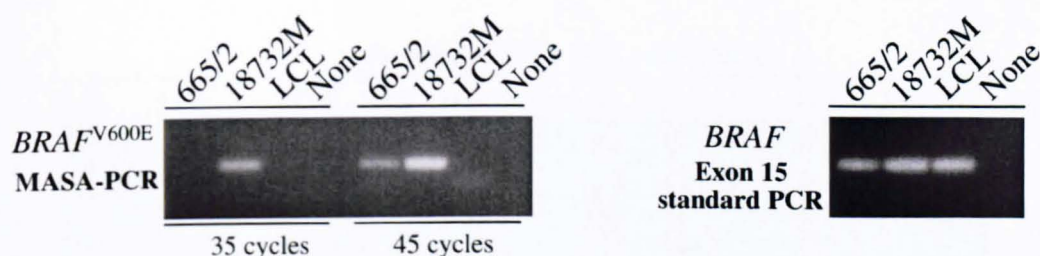
In this view, all the amount of data obtained from the microarray analysis provides a useful source of potential candidate genes which can mediate cell behavior characteristics as important as cell proliferation and cell cycle progression. Therefore, from the obtained data sets, other possible molecules which can reveal a tumor suppressive function can be extrapolated. The identification of new mediators of proliferation arrest in melanoma cells may open the way to the discovery of novel strategies for innovative therapeutic approaches in melanoma treatment.

FIGURES OF CHAPTER 3

A



B



C

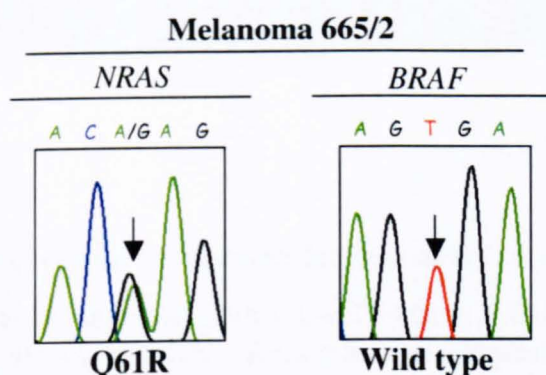


Figure 3.1. Detection of *NRAS*^{Q61R} and *BRAF*^{V600E} mutations.

A. Representative experiment performed on 5 *BRAF*^{V600E} (i.e. 10538P, 15392M, 4023M, 18732M, 18588M) and 2 *NRAS*^{Q61R} (i.e. 665/2, 8959M) tumors: amplification by MASA-PCR for the detection of *NRAS*^{Q61R} or *BRAF*^{V600E} mutations (upper panels) and by standard PCR for the exon 15 *BRAF* and exon 3 *NRAS* genes (lower panels). **B.** Amplification by 35- and 45- cycle MASA-PCR of *BRAF*^{V600E} (left panel) and amplification of exon 3 *BRAF* gene by standard PCR (right panel). **C.** Electropherograms of amplified exon 3 *NRAS* and exon 15 *BRAF* on melanoma 665/2 obtained by standard direct fluorescent sequencing.

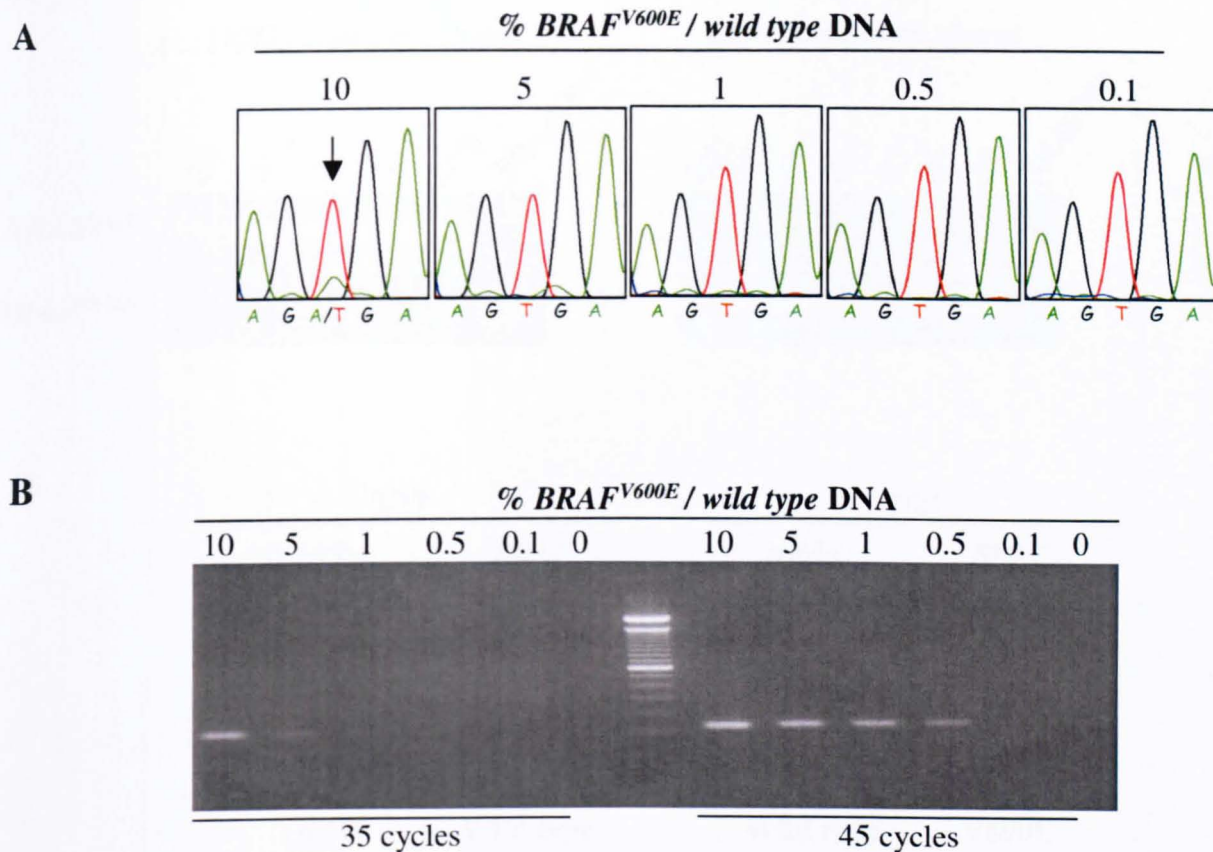


Figure 3.2. Titration assay for comparison between direct sequencing and MASA-PCR sensitivity.

Decreasing proportions of genomic DNA (10-0.1%) from *BRAF^{V600E}* melanoma 18732M were mixed with a fixed amount of genomic DNA from a human lymphoblastoid cell line (LCL) carrying only the wild type *BRAF* alleles. **A.** Electropherograms of titrated sample obtained by fluorescent direct sequencing. **B.** Amplification of titrated sample by 35- or 45- cycle MASA-PCR.

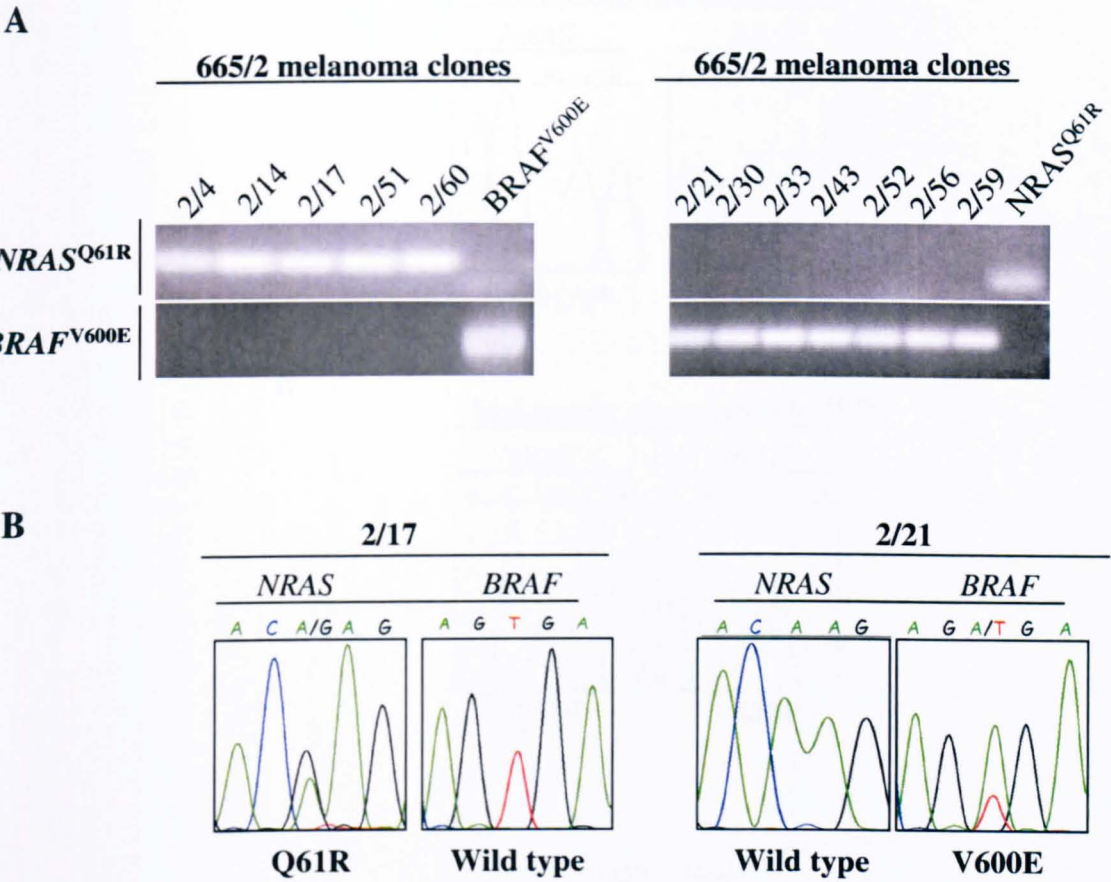


Figure 3.3. Mutational analysis of melanoma clones .

Representative results of clones isolated from a *NRAS*^{Q61R} metastatic lesion of patient 665/2. **A.** Amplification by MASA-PCR of either *NRAS*^{Q61R} or *BRAF*^{V600E} mutations. **B.** Electropherograms of amplified exon 3 *NRAS* and exon 15 *BRAF* obtained by standard direct fluorescent sequencing.

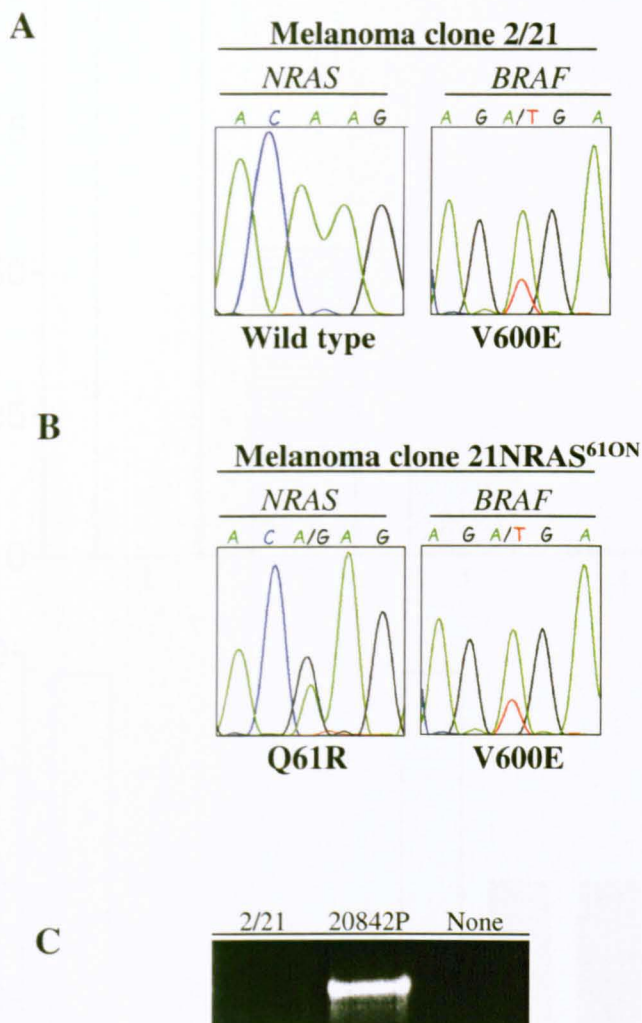


Figure 3.4. Detection of *NRAS* and *BRAF* mutations by direct sequencing in constitutive and inducible clones.

A. Electropherograms of partial exon 3 *NRAS* and exon 15 *BRAF* sequences amplified from genomic DNA of clone 2/21, showing a wild type homozygous *NRAS* sequence at codon 61 and A/T heterozygosity for *BRAF* at codon 600. **B.** Electropherograms displaying in 21NRAS^{61ON} the presence of either *NRAS* A/G heterozygosity at codon 61 as well as *BRAF* A/T heterozygosity at codon 600. **C.** Homozygous deletion of exon 3 of the *CDKN2A* locus in melanoma clone 2/21 evaluated by PCR; 20842P was used as positive control for *CDKN2A* locus amplification.

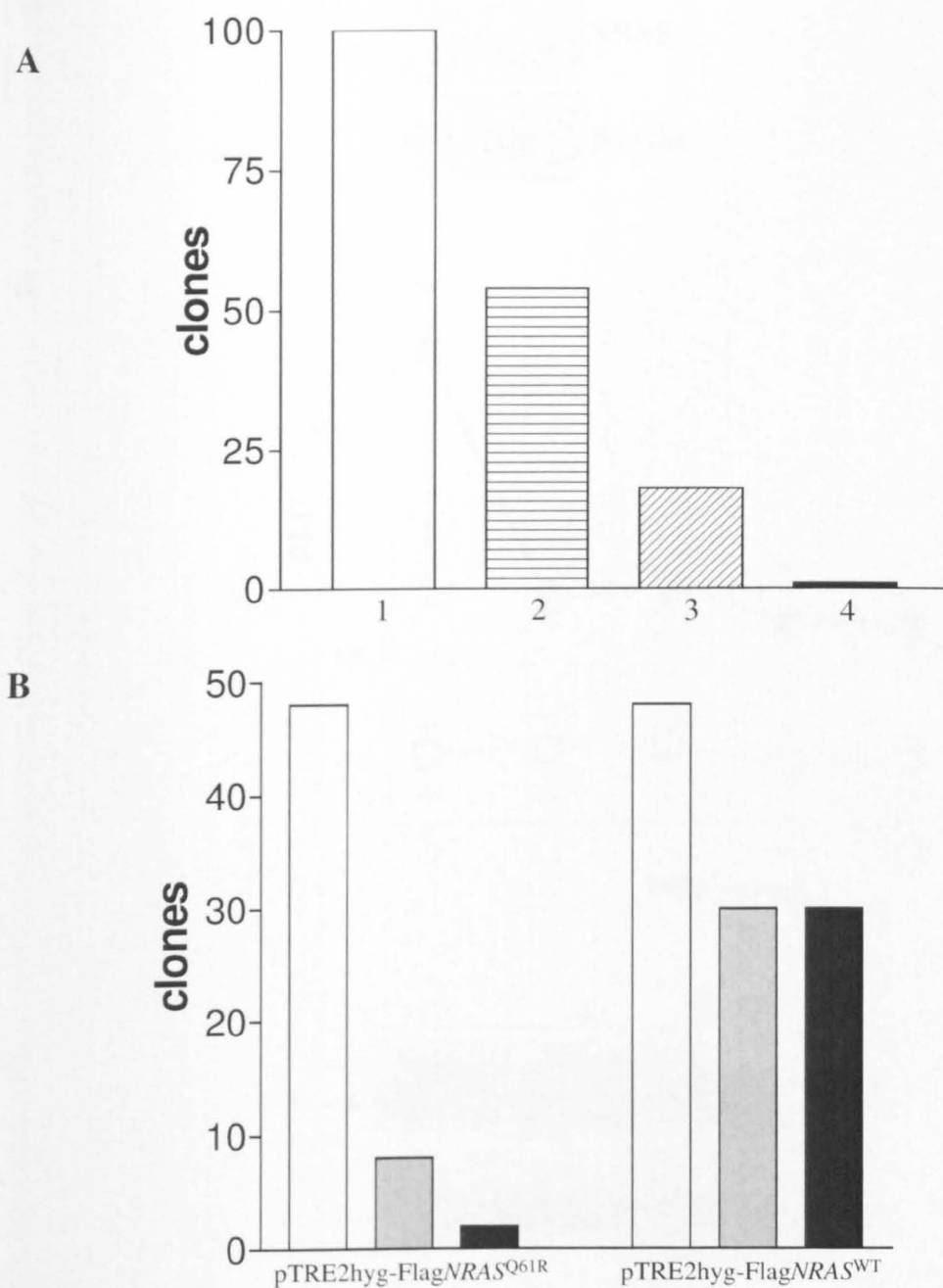


Figure 3.5. Selection of *NRAS*^{Q61R}/*BRAF*^{V600E} clones.

A. Number of G418 resistant clones isolated following transfection of pcDNA3.1-Flag/*NRAS*^{Q61R} (1), grown after selection (2), positive for the insert (3) and expressing the mutated transgene (4).

B. Number of clones isolated following transfection with the indicated expression vectors (white), after G418 selection (grey) or expressing the inducible transgene (black).

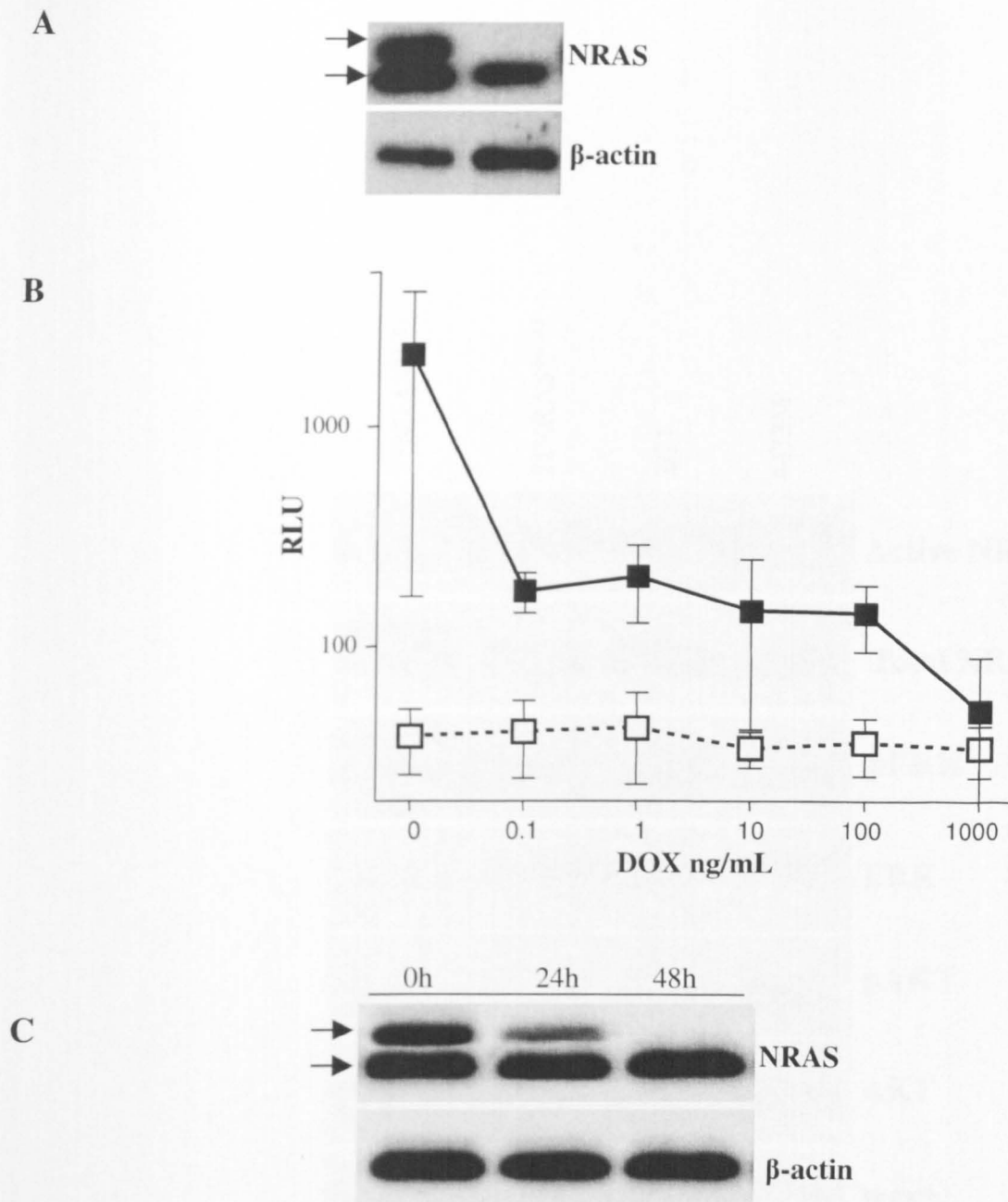


Figure 3.6. Constitutive and doxycycline-regulated expression of mutated $NRAS^{Q61R}$ in $BRAF^{V600E}$ melanoma clone 2/21.

A. Expression of Flag-tagged $NRAS^{Q61R}$ in 21 $NRAS^{Q61R}$ cells by Western Blot analysis using NRAS-specific mAb (upper arrow: exogenous Flag-tagged $NRAS^{Q61R}$; lower arrow: endogenous wild type NRAS). **B.** Luciferase activity of 21Off cells either untransfected (empty square) or transiently transfected with pTRE2hyg-*Luc* (black square) and cultured for 48 hours at 37°C in absence or in presence of increasing Dox concentrations. Results are expressed as Relative Light Units (RLU), where each value is the mean \pm s.d. of triplicate measurements. **C.** Time course silencing of $NRAS^{Q61R}$ transgene. 21 $NRAS^{Q61R}$ cells were cultured in the absence or with Dox (1 μ g/mL) for up to 48 hours. Cell lysates were then tested by Western blot analysis with an NRAS-specific mAb (upper arrows: exogenous Flag-tagged $NRAS^{Q61R}$; lower arrows: endogenous wild type NRAS). β -actin mAb was used as loading control.

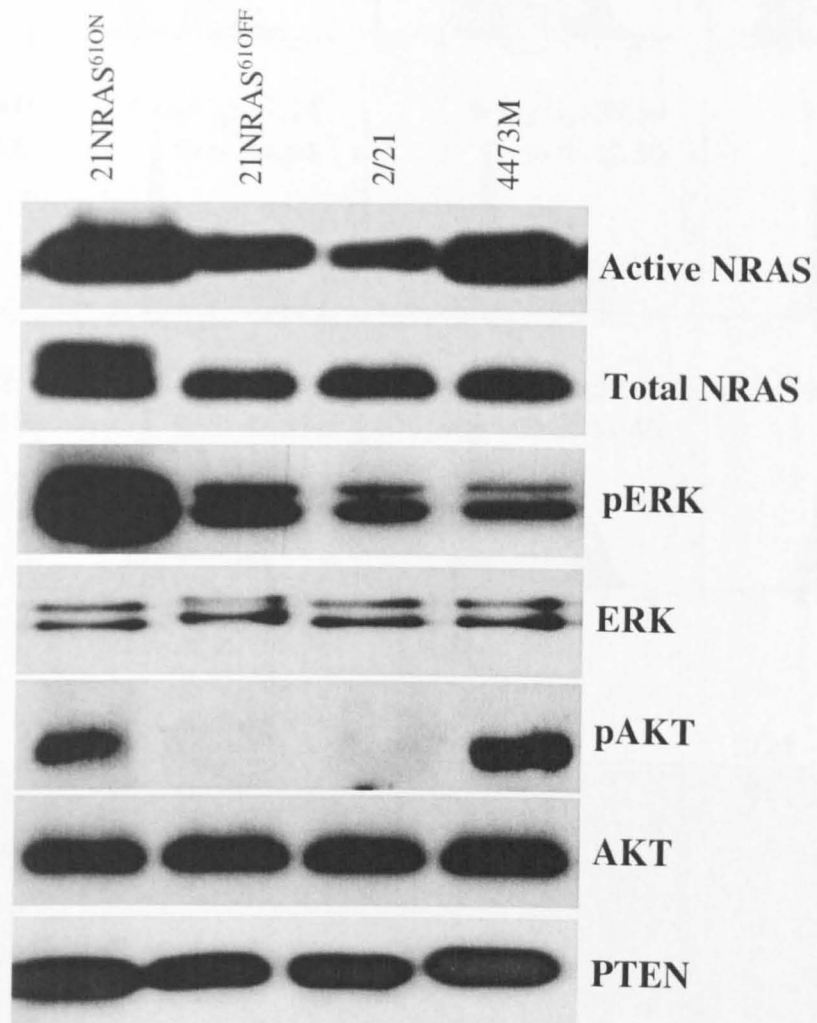
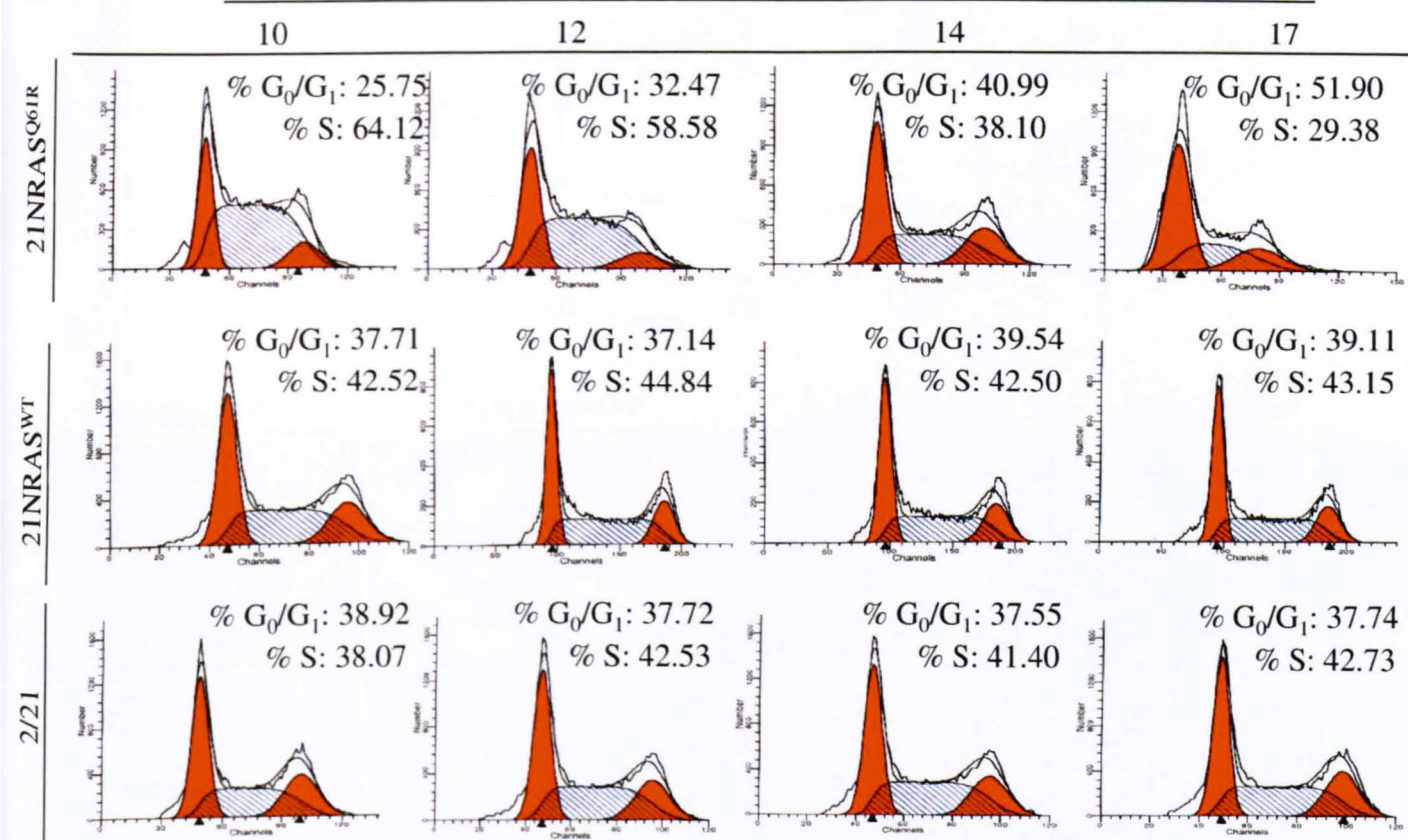


Figure 3.7. NRAS activation, ERK and AKT phosphorylation in 21NRAS^{61ON} cells.

Western blot analysis of active NRAS immunoprecipitated from 21NRAS^{61ON}, 21NRAS^{61OFF}, clone 2/21 (BRAF^{V600E}) and 4473M (NRAS^{Q61R}) melanoma cell lines, and of total NRAS, phosphorylated ERK (pERK), ERK, phosphorylated AKT (pAKT), AKT and PTEN from cell lysates of the same samples.

A

In vitro passages



B



Figure 3.8. Accumulation in the G₀/G₁ phase of the cell cycle and expression of a senescence-associated marker in BRAF^{V600E} melanoma cells with constitutive NRAS^{Q61R} expression.

A. Cell cycle analysis of 21NRAS^{Q61R} cells, compared to 21NRAS^{WT} and to parental 2/21 cells between the 10th and the 17th *in vitro* passage. **B.** SA-β-galactosidase staining of the parental 2/21 line, of 21NRAS^{Q61R} cells, and 21NRAS^{WT} (both at the 14th *in vitro* passage).

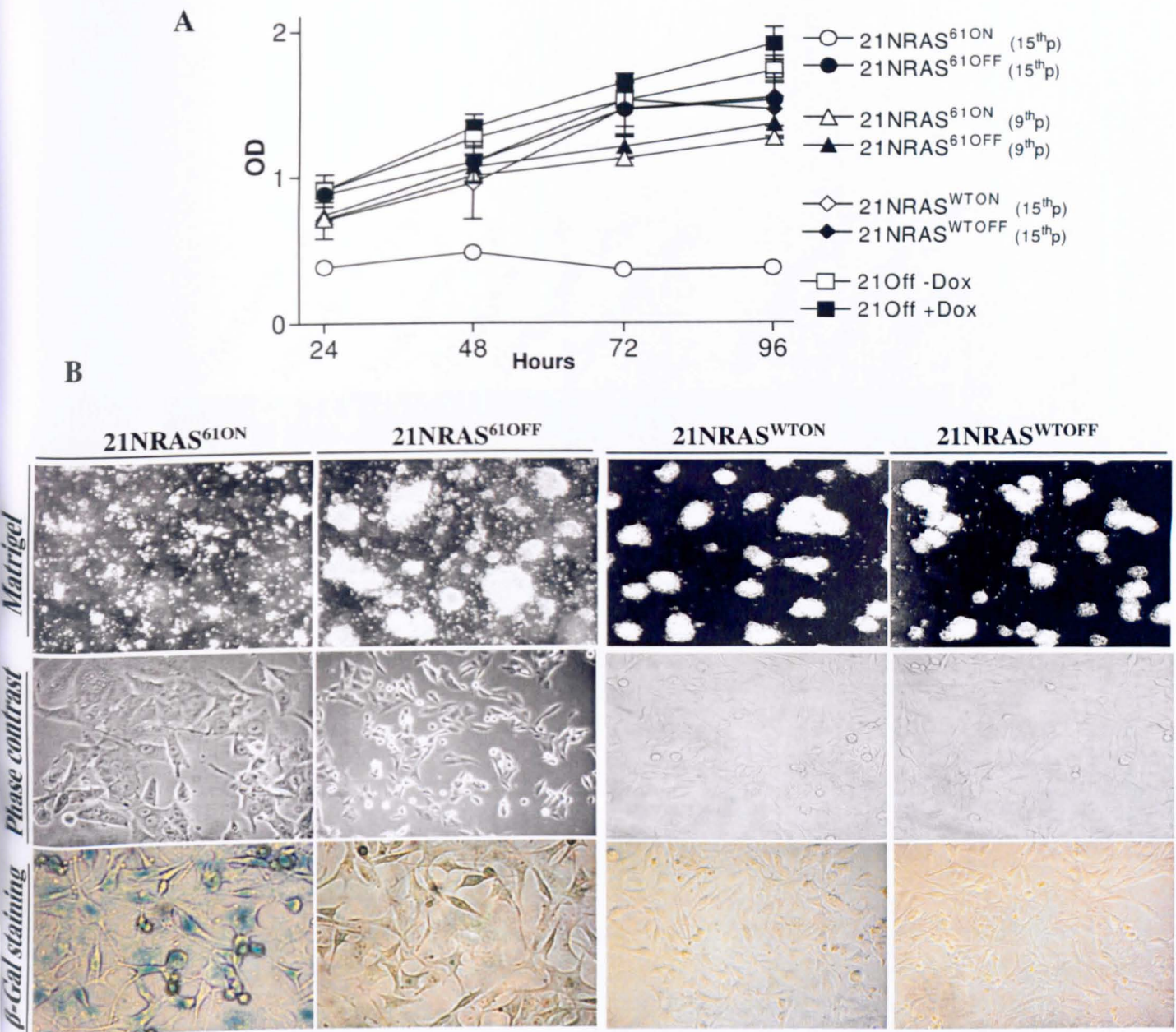


Figure 3.9. Differential growth characteristics and expression of senescence-associated markers in 21NRAS^{61ON} and 21NRAS^{61OFF} cells.

A. Proliferation, evaluated by MTS assay, of 21NRAS^{61ON} and 21NRAS^{61OFF}, at both the 9th and at the 15th *in vitro* passage, of 21NRAS^{WTON} and 21NRAS^{WTOFF} (at the 15th *in vitro* passage) and of 21Off cells cultured with/without Dox (1 µg/mL). OD_{490nm} mean ± s.d. of triplicates of one of three experiments are shown. OD_{490nm} values of 21NRAS^{61ON} at the 15th *in vitro* passage were significantly different at all time points from those of the other samples (ANOVA followed by SNK test, 0.001 < *p* < 0.05). **B.** Growth in Matrigel for one week of 21NRAS^{61ON} and 21NRAS^{61OFF} cells at the 15th *in vitro* passage (40x magnification). **C.** Growth on Matrigel support (upper panel), morphology (middle panel) and senescence-associated β-galactosidase activity (lower panel) of 21NRAS^{61ON} compared to 21NRAS^{61OFF} cells at the 15th *in vitro* passage.

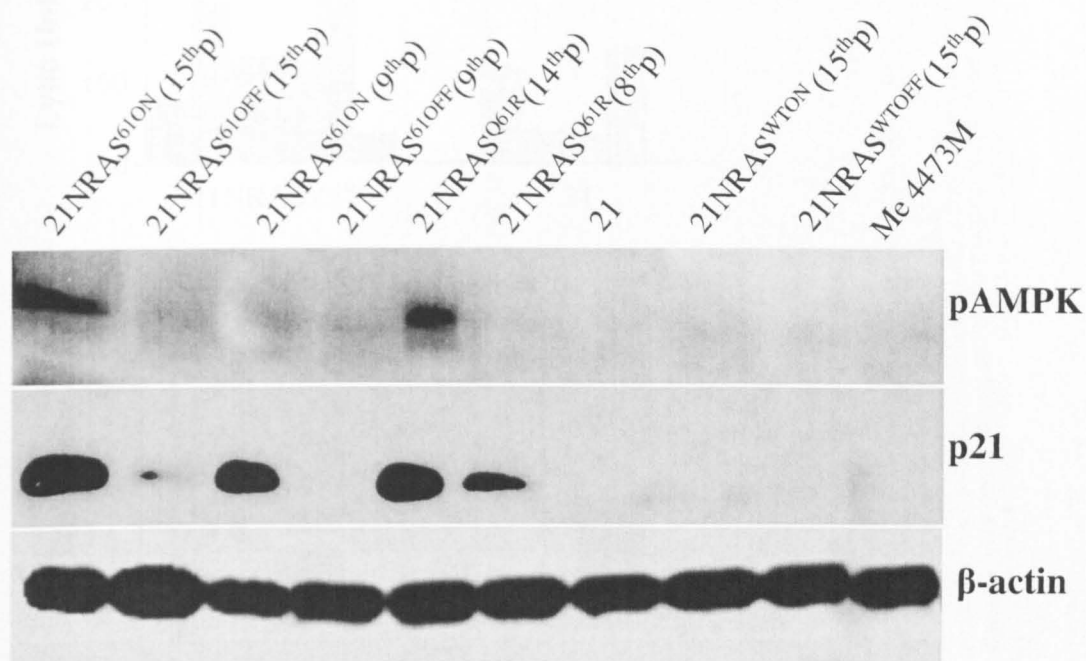


Figure 3.10. Expression of pAMPK and p21 after $NRAS^{Q61R}/BRAF^{V600E}$ co-expression.

Western Blot analysis of 21NRAS^{61ON} and 21NRAS^{61OFF} at both the senescent (15th) and non senescent (9th) *in vitro* passage, of constitutive transfectant 21NRAS^{Q61R} at both senescent (14th) and non senescent (8th) *in vitro* passage, of clone 2/21 ($BRAF^{V600E}$), of 21NRAS^{WTON} and 21NRAS^{WTOFF} at the 15th *in vitro* passage, and melanoma 4473M ($NRAS^{Q61R}$) for expression of phosphorylated AMPK (pAMPK), p21 and β-actin (as loading control).

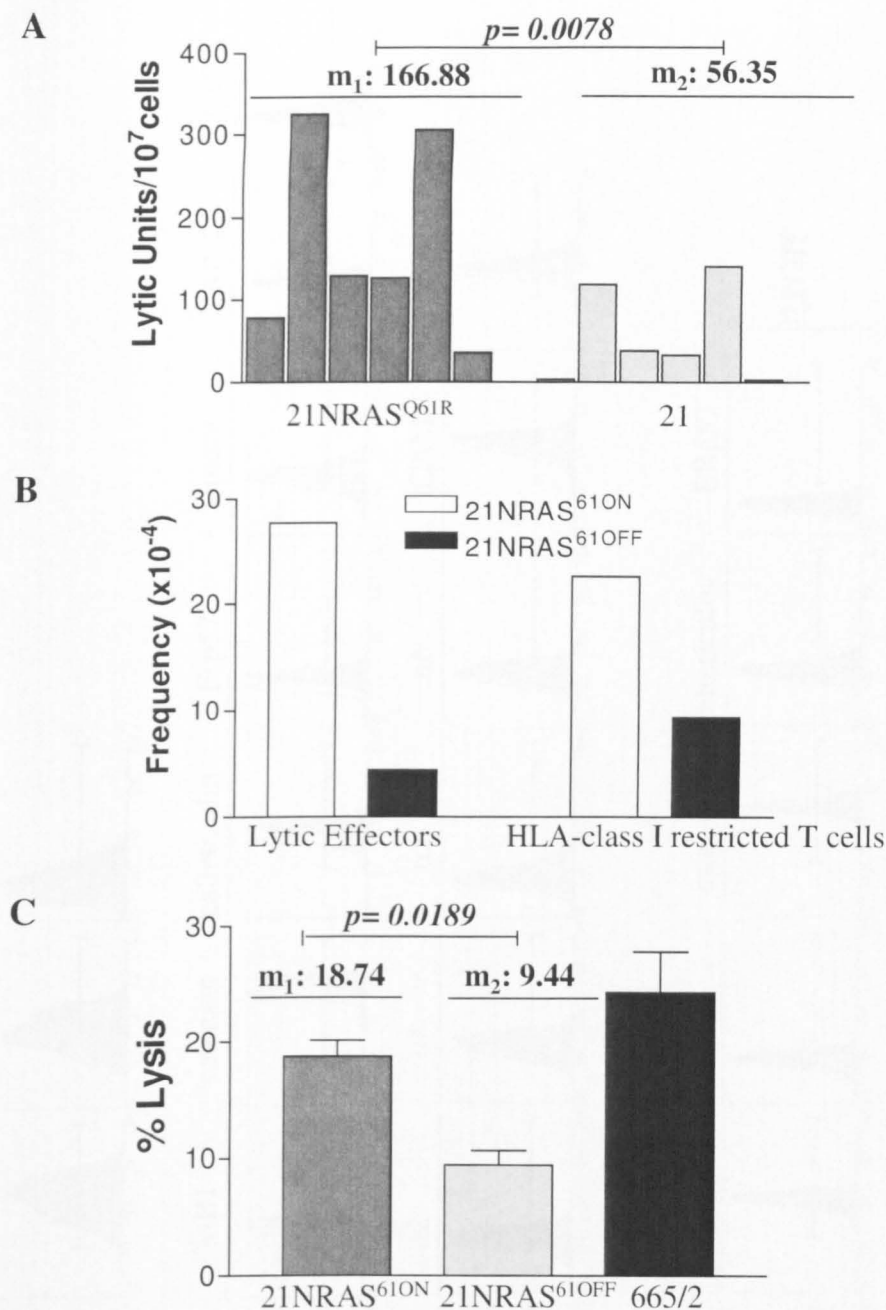


Figure 3.11. Increased susceptibility to cell mediated lysis of melanoma cells expressing constitutive or inducible NRAS^{Q61R}.

A. Lysis of 21NRAS^{Q61R} compared to clone 2/21 by LAK cells generated from six donors. Results expressed as lytic units (Lytic Units/ 10^7 lymphocytes, based on 30% target cell lysis). m_1 , m_2 : means of the six lytic unit values corresponding to 21NRAS^{Q61R} and 2/21 cells respectively; p : p-value obtained from a two-tailed t-test of the two data sets. **B.** Frequency of cytotoxic cells (lytic effectors) and of HLA-class I-restricted cytotoxic T cell effectors recognizing 21NRAS^{61ON} and 21NRAS^{61OFF} as evaluated by LDA with an alloreactive T cell line generated by MLTC against 21NRAS^{61OFF} cells. Frequency of cytotoxic effectors expressed as cytotoxic cells/ 10^4 lymphocytes. **C.** Lysis of 21NRAS^{61ON}, 21NRAS^{61OFF} and parental 665/2 tumor by the autologous HLA class I-restricted CTL clone 8B3. m_1 , m_2 : means of the % lysis relative to 21NRAS^{61ON}, 21NRAS^{61OFF} respectively; p : p-value obtained by a two-tailed t-test on the two data sets.

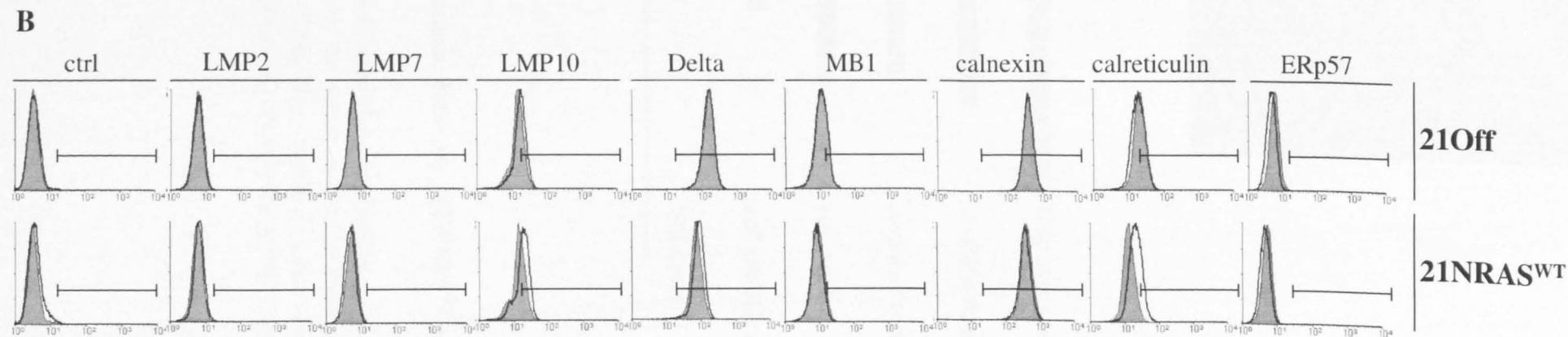
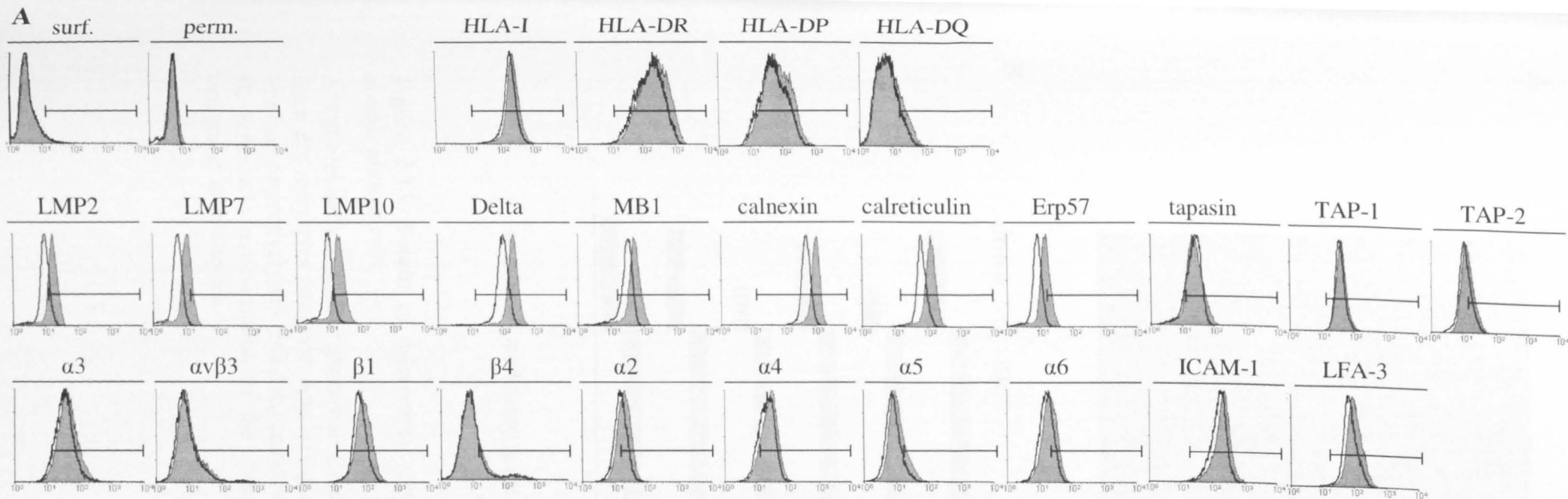
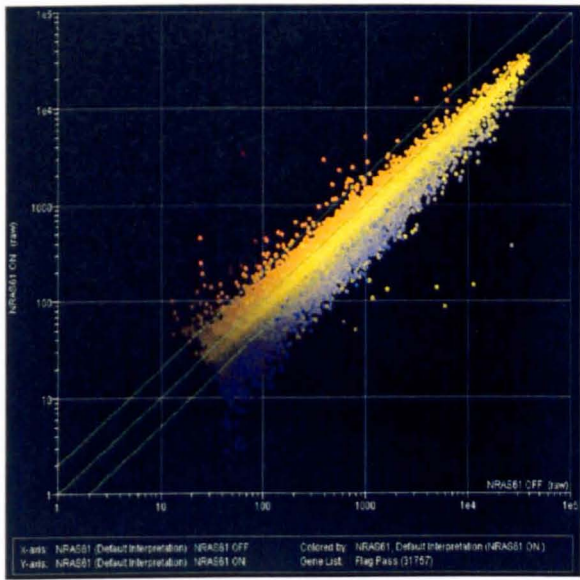


Figure 3.12. APM component up-regulation in *BRAF*^{V600E} melanoma cells expressing inducible *NRAS*^{Q61R}.

A. Flow cytometry analysis for cell-surface molecules (HLA class I and II antigens, $\alpha v \beta 3$ integrin, $\beta 1$, $\beta 4$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$ integrin subunits, ICAM-1 and LFA-3 adhesion molecules) and for intra-cellular APM components (delta, MB1, LMP2, LMP7, LMP10, TAP-1, TAP-2, calnexin, calreticulin, ERp57, tapasin) in 21NRAS^{61ON} cells (grey histograms) and 21NRAS^{61OFF} (empty histograms). surf.: non-permeabilized cells stained with secondary antibody only; perm.: permeabilized cells stained with secondary antibody only. **B.** Upper row: flow cytometry analysis in permeabilized cells for selected APM components in 21Off cells without Dox (grey histograms) or with 1 $\mu\text{g/mL}$ Dox (empty histograms). Lower row: flow cytometry analysis in permeabilized cells for selected APM components in 21NRAS^{WTON} (grey histograms) or 21NRAS^{WTOFF} (empty histograms). ctrl: cells stained with secondary antibody only.

A



B

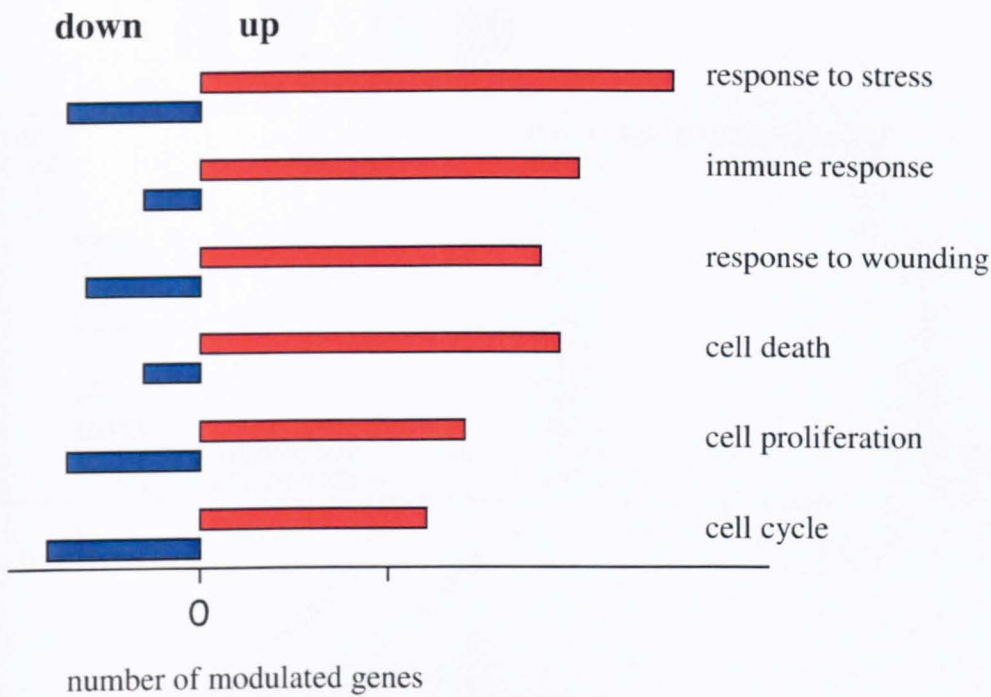


Figure 3.13. Results of Microarray comparison between 21NRAS^{61ON} and 21NRAS^{61OFF} double mutant cells.

A. Differentially expressed genes visualized as Scatter Plot by GeneSpring 7.0 software analysis. Each dot represents one gene; dots laying above the upper and below the lower diagonal green lines are respectively up- (red dots) and down- (blue dots) regulated genes of more than 1.5-fold.

B. Schematic representation of the distribution of modulated genes into the six reported categories of molecules.

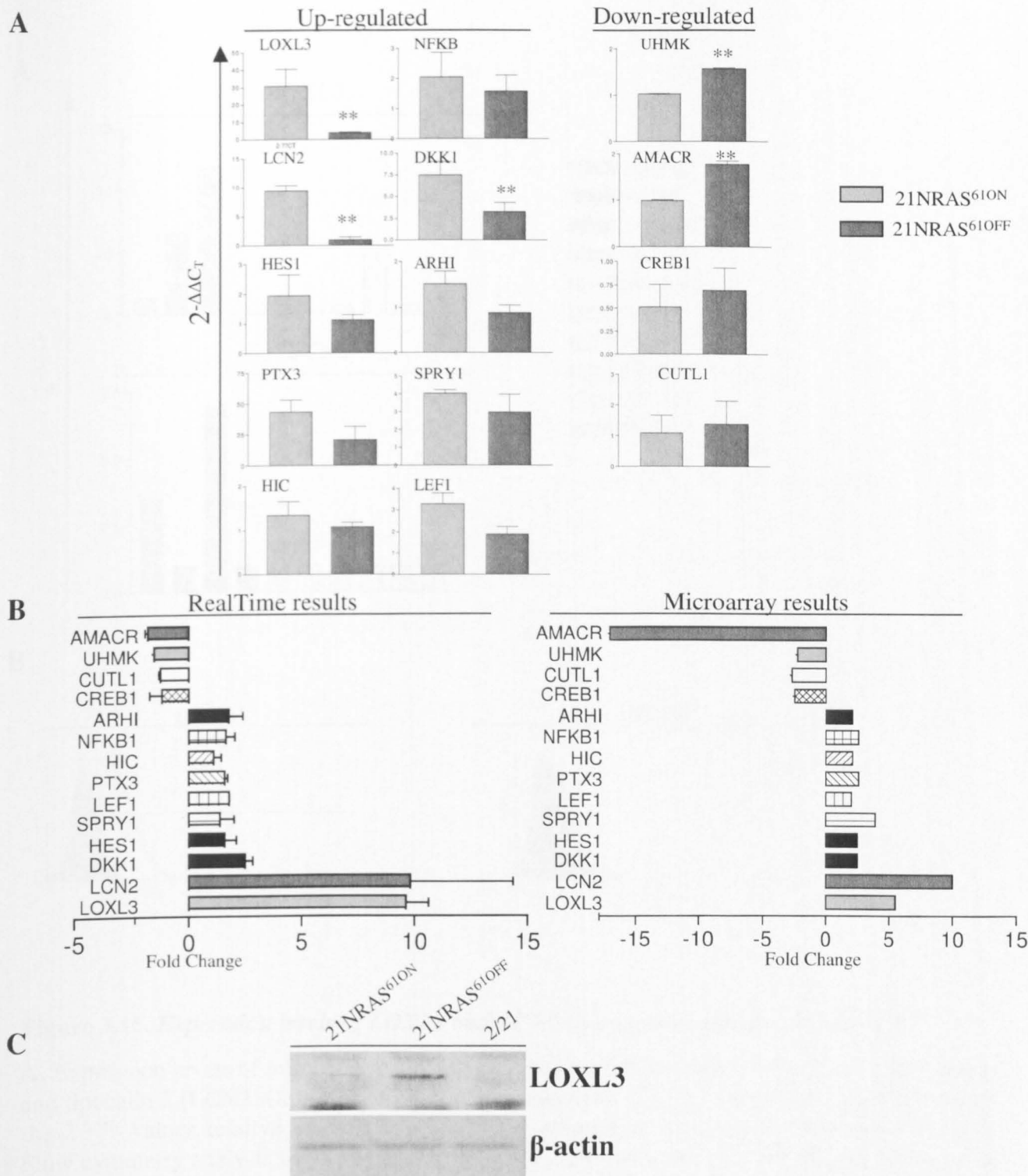
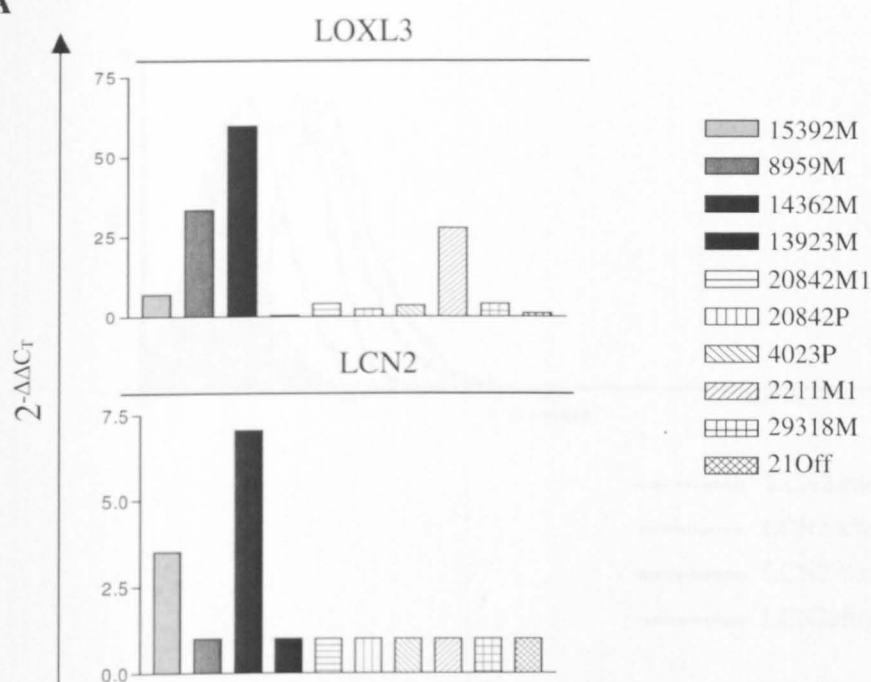


Figure 3.14. Validation of Microarray data by RealTime PCR.

A. Expression levels of mRNA of up- and down-regulated genes selected from microarray analysis as evaluated by RealTime PCR; the $2^{-\Delta\Delta C_T}$ values of 21NRAS^{61ON} (light grey) or 21NRAS^{61OFF} (dark grey) samples for each gene are represented in the histograms; $2^{-\Delta\Delta C_T}$: normalized target gene expression level. **: $0.05 > p > 0.001$ compared to NRAS^{61ON}. **B.** Comparison between fold change values obtained by RealTime validation and by Microarray experiment. RealTime results (left panel): fold changes correspond to the ratio between $2^{-\Delta\Delta C_T}$ values of 21NRAS^{61ON} and 21NRAS^{61OFF}; Microarray results: reported data correspond to the fold change values obtained in the data analysis by Gene Spring (see also Table 9). **C.** Protein expression levels of LOXL3 as evaluated by western Blot using a specific monoclonal antibody; β -actin was used as loading control.

A



B

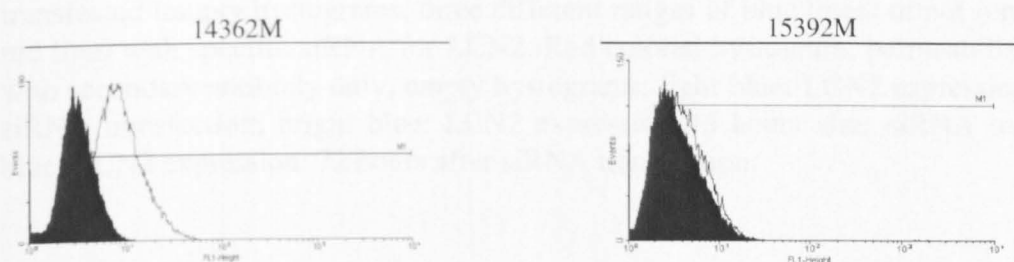


Figure 3.15. Expression levels of LOXL3 and LCN2 in a panel of melanoma cell lines.

A. Expression levels of mRNA as evaluated by RealTime PCR relative to LOXL3 (upper panel) and lipocalin 2 (LCN2) (lower panel) in a set of melanoma cell lines; each hystogram represents the $2^{-\Delta\Delta C_T}$ values relative to each sample. $2^{-\Delta\Delta C_T}$: normalized target gene expression level. **B.** Flow cytometry analysis for intra-cellular lipocalin 2 protein levels in 14362M cells (left panel) and 15392M (right panel). Gray hystograms: permeabilized cells stained with secondary antibody only; empty hystograms: permeabilized cells stained with both primary and secondary antibodies.

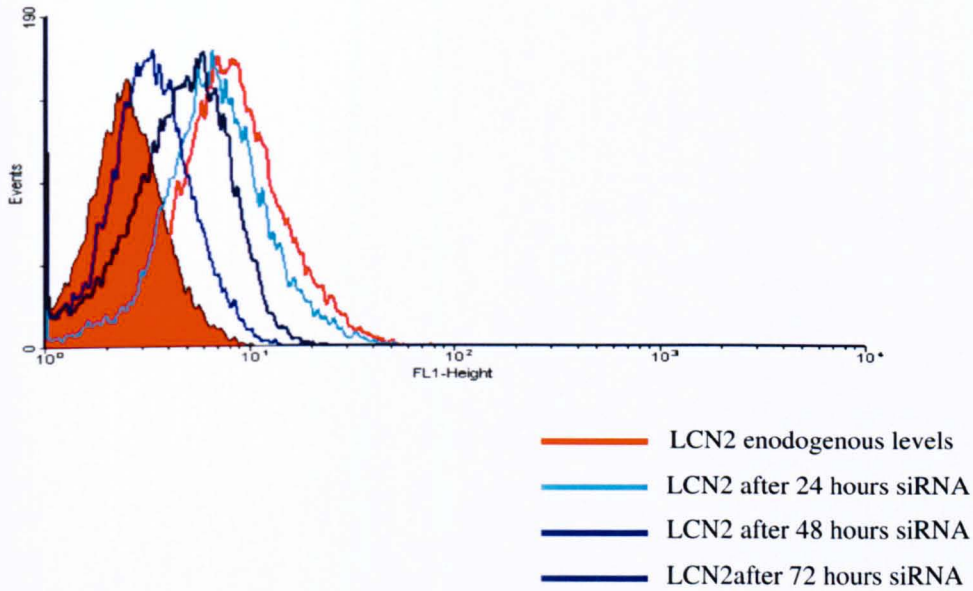


Figure 3.16. Silencing of LCN2 protein expression by siRNA.

Flow cytometry analysis for intra-cellular LCN2 protein levels in 14362M melanoma cells transfected (empty histograms, three different ranges of blue lines) or not (empty histogram, red line) with specific siRNA for LCN2. Red colored histogram: permeabilized cells stained with secondary antibody only; empty histograms: light blue: LCN2 expression 24 hours after siRNA transfection; bright blue: LCN2 expression 48 hours after siRNA transfection; dark blue: LCN2 expression 72 hours after siRNA transfection.

TABLES OF CHAPTER 3

Melanoma cell line	NRAS	BRAF
10538P	wt	V600E
15392M	wt	V600E
4023M	wt	V600E
18732M	wt	V600E
18588M	wt	V600E
4686M	wt	V600E
9923P	G12S	wt
4405P	Q61R	wt
8959M	Q61R	wt
5810P	Q61R	wt
4473P	Q61R	wt
665/2	Q61R	wt
665/1	Q61R	wt
665/R	Q61R	wt

Table 3.1: *NRAS* and *BRAF* mutations in the 14 melanoma cell lines analyzed (Daniotti e al., 2004).

	Number of mutated clones/total (%)			
	<i>BRAF</i> ^{V600E}	<i>NRAS</i> ^{Q61R}	<i>BRAF</i> ^{V600E} / <i>NRAS</i> ^{Q61R}	None
665/2	16/21 (75)	5/21 (25)	0/21 (0)	0/21 (0)
665/1	0/11 (0)	11/11 (100)	0/11 (0)	0/11 (0)
665/R	0/18 (0)	18/18 (100)	0/18 (0)	0/18 (0)
5810P	0/6 (0)	6/6 (100)	0/6 (0)	0/6 (0)

Table 3.2: *BRAF*^{V600E} and *NRAS*^{Q61R} mutations in clones isolated from 3 *NRAS*^{Q61R} metastatic lesions of the same patient (665/2, 665/1, 665/R) and from a different *NRAS*^{Q61R} patient (5810P).

Gene Symbol	Common Name	Fold Change
<i>BP75</i>	bromodomain containing protein 75 kDa human homolog	25.23
<i>LCN2</i>	lipocalin 2 (oncogene 24p3)	10
<i>PI3</i>	peptidase inhibitor 3, skin-derived (SKALP)	8
<i>IL1B</i>	interleukin 1beta	6.84
<i>TGM2</i>	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	6.13
<i>LOXL3</i>	lysyl oxidase-like 3	5.47
<i>PDLIM4</i>	PDZ and LIM domain 4	5.36
<i>CST7</i>	cystatin F (leukocystatin)	5.29
<i>NRAS</i>	neuroblastoma RAS viral (v-ras) oncogene homolog	5.19
<i>MGLL</i>	monoglyceride lipase	5.16
<i>KRT14</i>	keratin 14	4.72
<i>SLCO4C1</i>	solute carrier organic anion transporter family, member 4C1	4.55
<i>MGP</i>	matrix Gla protein	4.46
<i>NFATC2</i>	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	4.31
<i>FST</i>	folistatin	4.25
<i>MUC7</i>	mucin 7, salivary	4.15
<i>AREG</i>	amphiregulin (schwannoma-derived growth factor)	4.14
<i>MMP1</i>	matrix metalloproteinase 1 (interstitial collagenase)	4.13
<i>MB</i>	myoglobin	4.1
<i>SPRY1</i>	sprouty homolog 1, antagonist of FGF signaling	3.92
<i>COL6A3</i>	collagen, type VI, alpha 3	3.91
<i>CHERP</i>	calcium homeostasis endoplasmic reticulum protein	3.89
<i>PTPRE</i>	protein tyrosine phosphatase, receptor type, E	3.86
<i>TNA</i>	tetranectin (plasminogen binding protein)	3.83
<i>LAPTM5</i>	lysosomal associated multispinning membrane protein 5	3.74
<i>SYTL5</i>	synaptotagmin-like 5	3.69
<i>FAM20C</i>	family with sequence similarity 20, member C	3.68
<i>IER3</i>	immediate early response 3	3.63
<i>PLAUR</i>	plasminogen activator, urokinase receptor	3.55
<i>SLC20A1</i>	solute carrier family 20 (phosphate transporter), member 1	3.48
<i>SERPINE2</i>	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	3.46
<i>ENC1</i>	ectodermal-neural cortex (with BTB-like domain)	3.39
<i>RECQL5</i>	RecQ protein-like 5	3.38
<i>ARHI</i>	ras homolog gene family, member 1	3.32
<i>LNK</i>	lymphocyte adaptor protein	3.25
<i>UBCE7IP5</i>	likely ortholog of mouse ubiquitin conjugating enzyme 7 interacting protein 5	3.22
<i>LENG4</i>	leukocyte receptor cluster (LRC) member 4	3.21
<i>SPRR2C</i>	small proline-rich protein 2C	3.2
<i>VIPRI</i>	vasoactive intestinal peptide receptor 1	3.19
<i>IL11</i>	interleukin 11	3.14
<i>SERPINE1</i>	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	3.13
<i>TNC</i>	tenascin C (hexabrachion)	3.13
<i>C8FW</i>	phosphoprotein regulated by mitogenic pathways	3.12
<i>THBS1</i>	thrombospondin	3.04
<i>TERT</i>	telomerase reverse transcriptase	3.03

<i>TRAF1</i>	TNF receptor-associated factor 1	2.98
<i>NRG1</i>	neuregulin 1	2.97
<i>MDGA1</i>	MAM domain containing glycosylphosphatidylinositol anchor 1	2.97
<i>PHLDA2</i>	pleckstrin homology-like domain, family A, member 2	2.94
<i>HHIP</i>	hedgehog interacting protein	2.92
<i>MGC47816</i>	family with sequence similarity 80, member A	2.9
<i>SLC22A4</i>	solute carrier family 22 (organic cation transporter), member 4	2.89
<i>MRGX4</i>	MAS-related GPR, member X4	2.85
<i>ADORA3</i>	adenosine A3 receptor	2.84
<i>CDKN1A</i>	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	2.84
<i>AMPH</i>	amphiphysin (Stiff-Man syndrome with breast cancer 128kDa autoantigen)	2.81
<i>FOSL1</i>	fos-like antigen 1	2.78
<i>BIN3</i>	bridging integrator 3	2.75
<i>INHBA</i>	inhibin beta A (activin A, activin AB alpha polypeptide)	2.72
<i>CEGF3</i>	CUB domain and EGF-like repeat containing 3	2.7
<i>KCNN4</i>	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	2.68
<i>PTX3</i>	pentraxin-related gene, rapidly induced by IL-1 beta	2.63
<i>CHST2</i>	carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2	2.62
<i>TIMP3</i>	TIMP metalloproteinase inhibitor 3	2.61
<i>NFKB1</i>	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	2.6
<i>HR</i>	hairless homolog (mouse)	2.6
<i>DUSP6</i>	dual specificity phosphatase 6	2.57
<i>COL13A1</i>	collagen, type XIII, alpha 1	2.57
<i>DKK1</i>	dickkopf homolog 1 (<i>Xenopus laevis</i>)	2.51
<i>ENTPD7</i>	ectonucleoside triphosphate diphosphohydrolase 7	2.5
<i>HES1</i>	hairy and enhancer of split 1	2.49
<i>FGFRL1</i>	fibroblast growth factor receptor-like 1	2.49
<i>COL19A1</i>	collagen, type XIX, alpha 1	2.48
<i>FBXL14</i>	F-box and leucine-rich repeat protein 14	2.45
<i>NCR3</i>	natural cytotoxicity triggering receptor 3	2.42
<i>CCL20</i>	chemokine (C-C motif) ligand 20	2.42
<i>ADARB1</i>	adenosine deaminase, RNA-specific, B1 (RED1 homolog rat)	2.42
<i>CIAS1</i>	cold autoinflammatory syndrome 1	2.41
<i>MPZ</i>	myelin protein zero (Charcot-Marie-Tooth neuropathy 1B)	2.41
<i>KIF4A</i>	kinesin family member 4A	2.41
<i>EMILIN2</i>	elastin microfibril interfacer 2 1	2.4
<i>MID1</i>	midline 1 (Opitz/BBB syndrome)	2.39
<i>PDE2A</i>	phosphodiesterase 2A, cGMP-stimulated	2.38
<i>CPA3</i>	carboxypeptidase A3 (mast cell)	2.38
<i>RASD2</i>	RASD family, member 2	2.38
<i>PHLDA1</i>	pleckstrin homology-like domain, family A, member 1	2.37
<i>PFKFB4</i>	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	2.36
<i>LYZ</i>	lysozyme (renal amyloidosis)	2.33
<i>S100A7</i>	S100 calcium binding protein A7 (psoriasin 1)	2.32
<i>TGFB1</i>	transforming growth factor, beta-induced	2.31
<i>COL12A1</i>	collagen, type XII, alpha 1	2.31
<i>CA9</i>	carbonic anhydrase IX	2.31
<i>ATP2A2</i>	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	2.28

<i>ARAP3</i>	ARF-GAP, RHO-GAP, ankyrin repeat and plekstrin homology domains-containing protein 3	2.28
<i>ADAMTS5</i>	ADAM metalloproteinase with thrombospondin type 1 motif, 5 (aggrecanase-2)	2.21
<i>EEF1A2</i>	eukaryotic translation elongation factor 1 alpha 2	2.19
<i>UBE2D3</i>	ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	2.18
<i>ART3</i>	ADP-ribosyltransferase 3	2.18
<i>CTSE</i>	cathepsin E	2.16
<i>HIC</i>	l-mfa domain-containing protein	2.16
<i>IGF1</i>	insulin-like growth factor 1 (somatomedin C)	2.14
<i>GNAI3</i>	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3	2.14
<i>NTRK1</i>	neurotrophic tyrosine kinase, receptor, type 1	2.14
<i>PHLDA3</i>	pleckstrin homology-like domain, family A, member 3	2.13
<i>THAP10</i>	THAP domain containing 10	2.12
<i>SF3B4</i>	splicing factor 3b, subunit 4, 49kDa	2.11
<i>TIE</i>	tyrosine kinase with immunoglobulin and epidermal growth factor homology domains	2.11
<i>MSCP</i>	solute carrier family 25, member 37	2.1
<i>RIS1</i>	Ras-induced senescence 1	2.1
<i>SLC26A2</i>	solute carrier family 26 (sulfate transporter), member 2	2.1
<i>PRKDC</i>	protein kinase, DNA-activated, catalytic polypeptide	2.09
<i>CTGF</i>	connective tissue growth factor	2.08
<i>NOTCH2</i>	Notch homolog 2	2.08
<i>MYOZ2</i>	myozenin 2	2.07
<i>CHGN</i>	chondroitin beta1,4 N-acetylgalactosaminyltransferase	2.06
<i>SYT1</i>	synaptotagmin 1	2.06
<i>PTGFRN</i>	prostaglandin F2 receptor negative regulator	2.05
<i>GCM1</i>	glial cells missing homolog 1 (Drosophila)	2.05
<i>WNT5B</i>	wingless-type MMTV integration site family, member 5B	2.05
<i>ITGB3</i>	integrin beta3	2.05
<i>NES</i>	nestin	2.04
<i>SLC37A3</i>	solute carrier family 37 (glycerol-3-phosphate transporter), member 3	2.03
<i>IFNGR2</i>	interferon gamma receptor 2 (interferon gamma transducer 1)	2.02
<i>RGSS5</i>	regulator of G-protein signalling 5	2.02
<i>LEF1</i>	lymphoid enhancer-binding factor 1	2.02
<i>TGFA</i>	transforming growth factor, alpha	2.01
<i>FCER1G</i>	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	2
<i>ACTN1</i>	actinin, alpha 1	2
<i>APXL</i>	apical protein-like (Xenopus laevis)	2
<i>CIP98</i>	CASK-interacting protein CIP98	2
<i>HYPM</i>	Huntingtin interacting protein M	2
<i>DEFB124</i>	defensin, beta 124	1.99
<i>RW1</i>	RW1 protein	1.99
<i>DHX9</i>	DEAH (Asp-Glu-Ala-His) box polypeptide 9	1.98
<i>MSN</i>	moesin	1.98
<i>ARHE</i>	ras homolog gene family, member E	1.98
<i>ZNF161</i>	zinc finger protein 161	1.97
<i>BOK</i>	BCL2-related ovarian killer	1.97
<i>AK3</i>	adenylate kinase 3	1.97
<i>DKFZP434F0318</i>	hypothetical protein DKFZp434F0318	1.97
<i>PFN2</i>	profilin 2	1.96

<i>HRB2</i>	HIV-1 rev binding protein 2	1.96
<i>TNFRSF10B</i>	tumor necrosis factor receptor superfamily, member	1.95
<i>PHC1</i>	polyhomeotic-like 1 (Drosophila)	1.95
<i>RBM3</i>	RNA binding motif (RNPI, RRM) protein 3	1.95
<i>ZFP36</i>	zinc finger protein 36, C3H type, homolog (mouse)	1.95
<i>AFURS1</i>	ATPase family homolog up-regulated in senescence cells	1.95
<i>MLLT7</i>	myeloid/lymphoid or mixed-lineage leukemia	1.94
<i>SIPA1L2</i>	signal-induced proliferation-associated 1 like 2	1.94
<i>RAI3</i>	retinoic acid induced 3	1.93
<i>SIAT4</i>	sialyltransferase 4A (beta-galactoside alpha-2,3-sialyltransferase)	1.93
<i>UBE2E1</i>	ubiquitin-conjugating enzyme E2E 1 (UBC4/5 homolog, yeast)	1.92
<i>MCL1</i>	myeloid cell leukemia sequence 1 (BCL2-related)	1.9
<i>BHLHB2</i>	basic helix-loop-helix domain containing, class B, 2	1.9
<i>PSMD5</i>	proteasome (prosome, macropain) 26S subunit, non-ATPase, 5	1.9
<i>S100A4</i>	S100 calcium binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog)	1.9
<i>GAL</i>	galactosidase	1.89
<i>CCNE2</i>	cyclin E2	1.89
<i>CST6</i>	cystatin E/M	1.89
<i>LPL</i>	lipoprotein lipase	1.89
<i>S100A2</i>	S100 calcium binding protein A2	1.89
<i>SETBP1</i>	SET binding protein 1	1.89
<i>RAPGEF4</i>	Rap guanine nucleotide exchange factor (GEF) 4	1.88
<i>CYP26A1</i>	cytochrome P450, family 26, subfamily A, polypeptide 1	1.88
<i>PLOD</i>	procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase, Ehlers-Danlos syndrome type VI)	1.88
<i>FIBL-6</i>	hemicentin 1	1.87
<i>SLC15A4</i>	solute carrier family 15, member 4	1.87
<i>DC-TM4F2</i>	tetraspanin similar to TM4SF9	1.87
<i>NHS</i>	Nance-Horan syndrome (congenital cataracts and dental anomalies)	1.87
<i>EMP2</i>	epithelial membrane protein 2	1.86
<i>CAPN5</i>	calpain 5	1.86
<i>SYN3</i>	synapsin III	1.85
<i>HMGA2</i>	high mobility group AT-hook 2	1.85
<i>DGCR8</i>	DiGeorge syndrome critical region gene 8	1.85
<i>EIF4G2</i>	eukaryotic translation initiation factor 4 gamma, 2	1.84
<i>HIVEP3</i>	human immunodeficiency virus type I enhancer binding protein 3	1.82
<i>HTR7</i>	5-hydroxytryptamine (serotonin) receptor 7	1.81
<i>SLC39A12</i>	solute carrier family 39 (zinc transporter), member 12	1.81
<i>GPR101</i>	G protein-coupled receptor 101	1.8
<i>SAA1</i>	serum amyloid A1	1.79
<i>PPP1R15A</i>	protein phosphatase 1, regulatory (inhibitor) subunit	1.78
<i>ADSS</i>	adenylosuccinate synthase	1.76
<i>FAT</i>	FAT tumor suppressor homolog 1 (Drosophila)	1.74
<i>DPF1</i>	D4, zinc and double PHD fingers family 1	1.71
<i>SDCCAG33</i>	serologically defined colon cancer antigen 33	1.71
<i>POPDC2</i>	popeye domain containing 2	1.71
<i>HNRPA1</i>	heterogeneous nuclear ribonucleoprotein A1	1.7
<i>PRPS2</i>	phosphoribosyl pyrophosphate synthetase 2	1.69
<i>YT521</i>		1.69

<i>BIRC6</i>	baculoviral IAP repeat-containing 6 (apollon)	1.53
<i>SIRT4</i>	sirtuin (silent mating type information regulation 2 homolog) 4	-1.78
<i>WARS2</i>	tryptophanyl tRNA synthetase 2 (mitochondrial)	-1.85
<i>RNF149</i>	ring finger protein 149	-1.88
<i>NME5</i>	non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)	-1.88
<i>CARD15</i>	caspase recruitment domain family, member 15	-1.88
<i>TBX18</i>	T-box 18	-1.88
<i>RBM4</i>	RNA binding motif protein 4	-1.88
<i>SRP14</i>	signal recognition particle 14kDa (homologous Alu RNA binding protein)	-1.88
<i>ITGB1BP1</i>	integrin beta 1 binding protein 1	-1.88
<i>ABHD2</i>	abhydrolase domain containing 2	-1.88
<i>CHRNA3</i>	cholinergic receptor, nicotinic, beta polypeptide 3	-1.92
<i>RPL21</i>	ribosomal protein L21	-1.92
<i>RPL21</i>	ribosomal protein L21	-1.92
<i>MT2A</i>	metallothionein 2A	-1.92
<i>HIST1H2BI</i>	synonyms: H2B/k, H2BfK; Homo sapiens histone 1, H2bi (HIST1H2BI), mRNA.	-1.92
<i>TUBD1</i>	tubulin, delta 1	-1.96
<i>STAU</i>	staufen, RNA binding protein (Drosophila)	-1.96
<i>BTN3A2</i>	butyrophilin, subfamily 3, member A2	-1.96
<i>CDC42</i>	CDC42 binding protein kinase alpha (DMPK-like)	-2
<i>RPL10</i>	ribosomal protein L10	-2
<i>HIBCH</i>	3-hydroxyisobutyryl-Coenzyme A hydrolase	-2
<i>IFITM1</i>	interferon induced transmembrane protein 1	-2.04
<i>CDH23</i>	cadherin-like 23	-2.04
<i>LGP2</i>	likely ortholog of mouse D11Jgp2	-2.04
<i>HIAN3</i>	human immune associated nucleotide 6	-2.04
<i>IRF2BP2</i>	interferon regulatory factor 2 binding protein 2	-2.04
<i>RAPGEF1</i>	Rap guanine nucleotide exchange factor (GEF) 1	-2.04
<i>ZNF434</i>	zinc finger protein 434	-2.08
<i>COBL</i>	cordon-bleu homolog (mouse)	-2.08
<i>FAM31C</i>	family with sequence similarity 31, member C	-2.08
<i>HLXB9</i>	homeo box HB9	-2.12
<i>MLLT7</i>	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 7	-2.12
<i>RPS23</i>	ribosomal protein S23	-2.12
<i>ZNF222</i>	zinc finger protein 222	-2.12
<i>PCDHGC3</i>	protocadherin gamma subfamily C, 3	-2.12
<i>NACA</i>	nascent-polypeptide-associated complex alpha polypeptide	-2.12
<i>JMJD3</i>	jumonji domain containing 3	-2.12
<i>CPEB2</i>	cytoplasmic polyadenylation element binding protein 2	-2.12
<i>TM4SF4</i>	transmembrane 4 L six family member 4	-2.17
<i>MAX</i>	MYC associated factor X	-2.17
<i>HIST2H3C</i>	Histone 2, H3c (HIST2H3C)	-2.17
<i>MTF</i>	mitochondrial ferritin	-2.17
<i>UPF3B</i>	UPF3 regulator of nonsense transcripts homolog B (yeast)	-2.22
<i>ACAT1</i>	acetyl-Coenzyme A acetyltransferase 1 (acetoacetyl Coenzyme A thiolase)	-2.22
<i>HPGD</i>	hydroxyprostaglandin dehydrogenase 15-(NAD)	-2.22
<i>PRG-3</i>	plasticity related gene 3	-2.22
<i>UBE2V2</i>	ubiquitin-conjugating enzyme E2 variant 2	-2.27

<i>DDX42</i>	DEAD (Asp-Glu-Ala-Asp) box polypeptide 42	- 2.27
<i>KIS</i>	kinase interacting with leukemia-associated gene (stathmin)	- 2.27
<i>FUT10</i>	fucosyltransferase10	- 2.32
<i>CD33</i>	CD33 antigen (gp67)	- 2.32
<i>MRPL51</i>	mitochondrial ribosomal protein L51	- 2.32
<i>AEBP2</i>	AE binding protein 2	- 2.32
<i>AAK1</i>	AP2 associated kinase 1	- 2.32
<i>FES</i>	feline sarcoma oncogene	- 2.38
<i>TUBE1</i>	tubulin, epsilon 1	- 2.38
<i>ZNF347</i>	zinc finger protein 347	- 2.38
<i>COX7B2</i>	cytochrome c oxidase subunit VIIb2	- 2.38
<i>STMN1</i>	stathmin 1/oncoprotein 18	- 2.38
<i>RAB4A</i>	RAB4A, member RAS oncogene family	- 2.38
<i>BPIL1</i>	bactericidal/permeability-increasing protein-like 1	- 2.38
<i>RPL10L</i>	ribosomal protein L10-like	- 2.43
<i>RPL36AL</i>	ribosomal protein L36a-like	- 2.43
<i>HIST1H4D</i>	histone 1, H4d	- 2.43
<i>GPR2</i>	G protein-coupled receptor 2	- 2.43
<i>SNAPC5</i>	small nuclear RNA activating complex, polypeptide 5, 19kDa	- 2.5
<i>CREB1</i>	cAMP responsive element binding protein 1	- 2.5
<i>SCO1</i>	SCO cytochrome oxidase deficient homolog 1 (yeast)	- 2.5
<i>CECR5</i>	cat eye syndrome chromosome region, candidate 5	- 2.5
<i>SNX12</i>	sorting nexin 12	- 2.5
<i>ZNF263</i>	zinc finger protein 263	- 2.56
<i>TRPC1</i>	transient receptor potential cation channel, subfamily C, member 1	- 2.56
<i>WBP11</i>	WW domain binding protein 11	- 2.56
<i>KRTHA1</i>	keratin, hair, acidic, 1	- 2.63
<i>NJMU-R1</i>	protein kinase Njmu-R1	- 2.63
<i>COX6C</i>	cytochrome c oxidase subunit VIc	- 2.63
<i>CGI-38</i>	brain specific protein	- 2.63
<i>KPNB3</i>	karyopherin (importin) beta 3	- 2.63
<i>THAP9</i>	THAP domain containing 9	- 2.63
<i>RPS7</i>	ribosomal protein S7	- 2.70
<i>LGALS3</i>	lectin, galactoside-binding, soluble, 3 (galectin 3)	- 2.70
<i>RPP38</i>	ribonuclease P/MRP 38kDa subunit	- 2.70)
<i>TTC12</i>	tetratricopeptide repeat domain 12	- 2.70
<i>NARG7</i>	NMDA receptor-regulated gene 2	- 2.70
<i>NAT5</i>	N-acetyltransferase 5 (ARD1 homolog, <i>S. cerevisiae</i>)	- 2.70
<i>AHSG</i>	alpha-2-HS-glycoprotein	- 2.77
<i>H41</i>	hypothetical protein H41	- 2.77
<i>CUTL1</i>	cut-like 1, CCAAT displacement protein (<i>Drosophila</i>)	- 2.77
<i>CDT1</i>	DNA replication factor	- 2.77
<i>RASGRP1</i>	RAS guanyl releasing protein 1 (calcium and DAG-regulated)	- 2.77
<i>NEK1</i>	NIMA (never in mitosis gene a)-related kinase 1	- 2.77
<i>CALM2</i>	calmodulin 2 (phosphorylase kinase, delta)	- 2.77
<i>PEX7</i>	peroxisomal biogenesis factor 7	- 2.77
<i>DNAJA1</i>	DnaJ (Hsp40) homolog, subfamily A, member 1	- 2.85
<i>ZP4</i>	zona pellucida glycoprotein 4	- 2.85

<i>RPL26</i>	ribosomal protein L26	- 2.85
<i>TXNRD1</i>	thioredoxin reductase 1	- 2.85
<i>SIPA1L1</i>	signal-induced proliferation-associated 1 like 1	- 2.85
<i>LRRC2</i>	leucine rich repeat containing 2	- 2.85
<i>ATRN</i>	attractin	- 2.94
<i>RPL17</i>	ribosomal protein L17	- 2.94
<i>RPL17</i>	ribosomal protein L17	- 2.94
<i>MIZF</i>	MBD2 (methyl-CpG-binding protein)-interacting zinc finger protein	- 2.94
<i>CASC1</i>	cancer susceptibility candidate 1	- 2.94
<i>PLAC9</i>	placenta-specific 9	- 2.94
<i>SLC6A13</i>	solute carrier family 6 (neurotransmitter transporter, GABA), member 13	- 2.94
<i>SRD5A1</i>	steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)	- 3.03
<i>USP16</i>	ubiquitin specific peptidase 16	- 3.03
<i>CNGA3</i>	cyclic nucleotide gated channel alpha 3	- 3.12
<i>HSD17B7</i>	hydroxysteroid (17-beta) dehydrogenase 7	- 3.22
<i>HIMAP2</i>	immunity associated protein 2	- 3.22
<i>STK22D</i>	serine/threonine kinase 22D (spermiogenesis associated)	- 3.22
<i>ZNF15L1</i>	zinc finger protein 708 (KOX8)	- 3.33
<i>ARID4B</i>	AT rich interactive domain 4B (RBPI- like)	- 3.33
<i>TNNT1</i>	troponin T1, skeletal, slow	- 3.44
<i>GLS</i>	glutaminase	- 3.44
<i>SWAP70</i>	SWAP-70 protein	- 3.44
<i>ZNF19</i>	zinc finger protein 19 (KOX 12)	- 3.70
<i>IL15</i>	interleukin 15	- 3.84
<i>SPA17</i>	sperm autoantigenic protein 17	- 3.84
<i>GALNT13</i>	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 13 (GalNAc-T13)	- 4
<i>P29</i>	GCIP-interacting protein p29 isoform 1	- 4
<i>SST</i>	somatostatin	- 4.16
<i>PSIP1</i>	PC4 and SFRS1 interacting protein 1	- 4.16
<i>ZNF237</i>	zinc finger protein 237	- 6.25
<i>APOBEC3G</i>	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G	- 7.14
<i>PVALB</i>	parvalbumin	- 7.14
<i>TTL1</i>	tubulin tyrosine ligase-like family, member 1	- 8.33
<i>HIST1H2BG</i>	histone 1, H2bg	- 9.09
<i>TMOD2</i>	tropomodulin 2 (neuronal)	- 12.5
<i>AMACR</i>	alpha-methylacyl-CoA racemase	- 16.66

Table 3.3. List of the 321 modulated genes resulting from the MicroArray comparison between 21NRAS^{61ON} and 21NRAS^{61OFF}. Fold changes are calculated as the ratio of the raw values 21NRAS^{61ON}/21NRAS^{61OFF}.

IV. AMPK ROLE IN MELANOMA.

4.1 Introduction

The AMP-activated protein kinase (AMPK) is a fuel-sensing enzyme with a major role in the regulation of cellular lipid and protein metabolism. It is activated under conditions that deplete cellular ATP and elevate AMP levels leading to the rise of AMP:ATP ratio, such as glucose deprivation, heat shock, hypoxia and ischemia (Davies et al., 1989; Kemp et al., 2003), and also by hormones such as leptin (Minokoshi et al., 2002), adiponectin (Yamauchi et al., 2002), catecholamine (Ruderman et al., 2003), and interleukin-6 (Kelly et al., 2004). AMPK is activated through phosphorylation by at least two upstream AMP-activated protein kinase kinase (AMPKK) which are the tumor suppressor LKB1 (Hawley et al., 2003) and the Ca^{2+} /calmodulin-dependent protein kinase kinase (CaMKK) (Hurley et al., 2005). Once activated, AMPK switches on catabolic pathways, which generate ATP while switching off ATP-consuming processes. Mammalian AMPK is a heterotrimer complex containing the catalytic α - subunit and the regulatory β - and γ - subunits (Hardie et al., 2003.). Each of these subunits exists in at least two isoforms ($\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, $\gamma 1$, $\gamma 2$, $\gamma 3$) encoded by distinct genes (Figure 4.1). The $\alpha 1$ and $\alpha 2$ subunits contain a conventional kinase domain at the N-terminus while the C-terminus regions are required to form a complex with the β and the γ subunits (Figure 4.1) (Crute et al., 1998). The β -subunits contain two conserved regions: the ASC domain, which is involved in the interaction with α and γ subunits, and the KIS domain, which is a glycogen-binding domain (GBD). The γ subunit contain four tandem repeats of the

cystathionine β -synthase (CBS) sequence motif (Bateman, 1997), which represent the regulatory AMP- and ATP- binding sites of the AMPK complexes (Scott et al., 2004). A diverse array of different AMPK complexes arises from the possible combinations of the isoforms along with splice variants and the use of different promoters.

The activation of AMPK through 5'-AMP can occur in three different ways, all of which are antagonized by high ATP concentrations. First, AMP binding causes allosteric activation of mammalian AMPK with a magnitude of this effect variable between the different AMPK isoforms. Complexes containing the $\alpha 1$ and $\alpha 2$ subunits, which differ in their subcellular localization, have also different dependence on AMP activation: complexes containing the $\alpha 2$ subunit are partly localized in the nucleus and are activated 5- to 6- fold by AMP, whereas complexes with $\alpha 1$ are not in the nucleus and are only activated 1.5 to 2-fold by AMP (Salt et al., 1998). Second, AMP binding makes AMPK a better substrate for the upstream kinase. As previously shown (Hawley et al., 1996), the tumor suppressor LKB1 activates AMPK by phosphorylation of α subunit at a specific threonine residue (Thr172) within the activation loop domain. The Thr172 phosphorylation causes at least 50- to 100- fold activation and it is stimulated by AMP while it is inhibited by high ATP concentrations. AMP stimulation occurs only if the intact $\alpha\beta\gamma$ complex, and not the isolated kinase domain which lacks the AMP-binding sites, is used as the substrate (Hawley et al., 2003). This shows that the effect is due to binding of AMP to the substrate AMPK and not to the upstream kinase. The third way through which AMP regulates AMPK activation is that AMP binding inhibits dephosphorylation of Thr172 by protein phosphatases (Davies et al., 1995).

Activation of AMPK through phosphorylation by an upstream protein kinase exerts different effects on various downstream targets, either by direct phosphorylation of metabolic enzymes or by acting on either gene expression or mRNA stability or protein translation. In general, activated AMPK can down-regulate biosynthetic pathways such as fatty acid and cholesterol biosynthesis, as well as can switch on catabolic pathways which generate ATP, such as fatty acid oxidation, glucose uptake and glycolysis (Figure 4.2). For example, AMPK upregulates the expression of the co-activator PGC1 α (Terada et al., 2002) which is involved in the increased expression of mitochondrial genes in muscles (Zong et al., 2002), yet it downregulates the transcription factors SREBP-1c (Zhou et al., 2001) and HNF4 α (Leclerc et al., 2001) which are related to the decreased expression of genes involved in lipogenesis, glucose uptake and glycolysis in liver. In addition, AMPK phosphorylates the ubiquitous co-activator p300 at Ser89, reducing its ability to bind to nuclear hormone receptors and thus activate their target genes (Yang et al., 2001).

AMPK activation can also affect protein expression by regulating mRNA stability. Although the mechanism is not yet defined, it has been shown that activated AMPK reduces cytoplasmatic levels of the RNA-binding protein HuR which stabilizes specific mRNAs in the cytoplasm by binding to their 3'-untranslated regions (Wang et al., 2002). Among HuR target mRNAs there are proteins, which regulate cell cycle like cyclin A, cyclin B1 and p21. It has been shown (Wang et al., 2003) that activation of AMPK by an elevated AMP:ATP ratio in senescent fibroblasts can explain many features of the senescent phenotype *in vitro*. In addition, inhibition of translation as a consequence of AMPK activity can occur by at least two mechanisms: phosphorylation and activation of elongation factor-2 kinase which stops the elongation step in translation (Horman et al.,

2002; Browne et al., 2004), and inhibition of the mammalian target of rapamycin (mTOR) pathway (Bolster et al., 2002; Kimura et al., 2003), which is a major positive stimulus for protein synthesis, cell growth and cell size.

Several recent findings report a link between AMPK and the growth and/or survival of cancer cells. The relationship between AMPK and cancer is due to the fact that many cancers are characterized by increased expression or activity of enzymes, like fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC) and mTOR, which are inhibited through phosphorylation by AMPK. Moreover, AMPK signaling involves two tumor suppressors: LKB1, upstream, and TSC2, downstream. LKB1 was first identified as the gene mutated in Peutz-Jeghers syndrome (PJS), an autosomal-dominant genetic disorder characterized by multiple hamartomatous polyps in the gastrointestinal tract and an increase risk of developing malignant tumors (Boudeau et al., 2003). The mechanism by which LKB1 suppresses tumorigenesis is unclear but, at least in part, appears to be mediated by inhibition of PI3K and mTOR. TSC2, also known as tuberlin, is phosphorylated by AMPK and negatively regulates protein synthesis by inhibiting mTOR (Inoki et al., 2003). In addition, TSC2 is inhibited through phosphorylation by AKT, leading to activation of mTOR and an increase of protein synthesis. The regulation of mTOR and TSC2 by AMPK might have important implications because the PI3K-AKT signaling pathway is constitutively active in many cancers, most notably those that have inactivating mutations of the PTEN gene. For these reasons, AMPK activation can be of relevant impact in cancer cells where PI3K-mTOR pathway is often dysregulated.

4.2 Results

4.2.1 AICAR and Phenformin activate and phosphorylate AMPK.

As presented in Chapter 3.2.2.3, Thr172-phosphorylated AMP-activated protein kinase (AMPK) was expressed in both senescent inducible 21NRAS^{61ON} (15th *in vitro* passage) and constitutive 21NRAS^{Q61R} (14th *in vitro* passage) “double mutant” cells, but was not detected in both pre-senescent 21NRAS^{61ON} (9th *in vitro* passage) and 21NRAS^{Q61R} (8th *in vitro* passage) as well as in 21NRAS^{61OFF} cells (9th and 15th *in vitro* passage). These data have suggested that AMPK may represent a possible mediator of proliferation arrest in human melanoma. Therefore, the role of AMPK molecule, previously shown as modulated in the double mutated model system, was further investigate in a panel of human melanoma cell lines in order to eventually identify possible mechanisms that might reactivate a senescent program in tumor cells.

5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) is a synthetic activator of AMPK. Upon entering the cell, AICAR is converted by adenosine kinase to ZMP, a cellular mimetic of AMP, which activates AMPK (Corton et al., 1995). Phenformin is a closely related analog of Metformin, which activates AMPK by an unknown mechanism likely not involving changes in intracellular levels of AMP or the ATP:ADP ratio (Hawley et al., 2002; Fryer et al., 2002).

In order to achieve exogenous activation of AMPK, melanoma cells were treated with different doses of either AICAR or Phenformin drugs. Increase of both kinase enzymatic activity and phosphorylation levels of AMPK as well as increase of phosphorylation levels of its downstream target acetyl-CoA carboxylase (ACC) were tested in those

samples. To this end, total AMPK protein was immunoprecipitated from melanoma cell lysates using antibodies against $\alpha 1$ and $\alpha 2$ subunits of the protein. Immunocomplexes were then tested by a specific kinase assay using the AMARA peptide (AMARAASAAALARRR) as a substrate of AMPK. As a consequence of either AICAR or Phenformin treatment, AMPK kinase activity was boosted. Representative results of the IP kinase assay in two melanoma cell lines (1402R, 1007P) treated or not with different doses of Phenformin are shown in Figure 4.3. AMPK kinase activity increased in a drug dose-dependent manner. The highest levels of AMPK activity could be achieved after 2 hour treatment with 5 mM Phenformin. Similar results were obtained in the same panel of melanoma cells after AICAR treatments with the same range of doses (data not shown). However, as Figure 4.3 shows, both basal and drug-induced kinase activities were lower in melanoma cells than in control cells, i.e. Hek293 cells treated or not with 5 mM Phenformin for 2 hours. This observation suggests that melanoma cells might have an intrinsic mechanism to prevent AMPK activation or to impair drug response in the cell.

In order to assess whether the observed enzymatic activation upon either AICAR or Phenformin treatment was concomitant with increment of AMPK phosphorylation levels, melanoma cells were treated with different doses of either drugs. In response to drug treatment, phosphorylation levels of both the AMPK α -subunit in Thr172 (pAMPK) and acetyl CoA-carboxylase in Ser79 (pACC) were evaluated by Western blot. Since ACC is a direct downstream target of AMPK, increment of pACC levels represents a direct measurement of AMPK phosphorylation state. As shown in Figure 4.4 A, pACC and pAMPK levels were up-regulated in 4405P after treatment with 5 mM AICAR for 2

hours. Increased expression levels of pAMPK and pACC are shown in 20842P and 1402R melanoma cells treated with 1 mM, 5 mM and 10 mM AICAR for 2 hours (Figure 4.4 A). Modulation of phosphorylation levels of both pACC and pAMPK was quantified by infra-red imaging using Li-Cor Odyssey system, as the ratio of the signal band intensity relative to the phosphorylated protein and the correspondent band relative to the total protein. Resultant values are visualized as histogram graphs (Figure 4.4 B). In each experiment, lysates from human liver cells treated or not with 5 mM AICAR were used as a control for increased phosphorylated ACC and AMPK expression. Other melanoma cell lines resulted similarly sensitive to 1 mM, 5 mM and 10 mM AICAR treatment for 2 hours. In addition, similar results were also obtained upon Phenformin treatment (data not shown).

Taken together, these data indicated that both AICAR and Phenformin compounds are effective in activating AMPK in melanoma cells in term of both enzymatic activity and phosphorylation levels.

4.2.2 Drug induced-AMPK activation impacts on cell proliferation in melanoma cells.

In vitro growth properties of melanoma cells after treatment with either AICAR or Phenformin differed from correspondent non treated cells. As evaluated by MTS/PMS test, OD_{490nm} of eight melanoma cell lines, human fibroblasts and normal melanocytes gradually decreased in dose- and time-dependent manner. In Figure 4.5 A growth curves relative to two representative melanoma cell lines are shown. 4405P and 20842P samples were treated with AICAR or Phenformin for 8 days. Since the 3rd day of treatment,

OD_{490nm} of melanoma cells cultured with 1 mM, 2 mM and 5 mM AICAR were significantly lower than non treated cells (Figure 4.5 A, left panel). Similar behavior was observed when cells were treated with Phenformin, even before the 3rd day (Figure 4.5 A, right panel). Interestingly AICAR and Phenformin at the concentrations of 1 mM, 2 mM and 5 mM induced progressive decrease of cell proliferation in normal human fibroblasts and in normal melanocytes as well (Figure 4.5 A and B, lowest panels), indicating that neoplastic cells retain responsiveness to AICAR and Phenformin as the normal cells. Maximal levels of cell proliferation inhibition, measured as the decrease of OD_{490nm}, were reached after 8 days of treatment with either AICAR or Phenformin. The reduction of OD_{490nm} was calculated for each dose of AMPK activators as the percentage of the ratio of OD_{490nm} values between treated and not treated cells. In the 8th day since treatment, proliferation rate in treated cells was significantly lower than in not treated cells. OD_{490nm} values decreased in dose-dependent manner, as summarized for two representative melanoma cell lines in Figure 4.5 B.

For both AICAR and Phenformin drugs, the effects of additional doses were verified. Lower doses such as 0.1 mM, 0.2 mM and 0.5 mM of both AMPK activators were tested and did not seem to affect proliferation rate of either melanoma cells or fibroblasts and normal melanocytes (Figure 4.6 A, for representative results relative to fibroblasts). On the other hand, concentrations higher than 5 mM of both drugs clearly induced cell detachment and apoptotic cell death (data not shown). Notably, melanoma cells treated with 1 mM AICAR remained viable, as assessed by the observation of trypan-blue colored cells, and did not die for apoptosis even after 8 days of treatment

(Figure 4.6 B). In contrast, the same dose of Phenformin was effective in inducing cell death by apoptosis and necrosis (Figure 4.6 B).

Taken together, these data showed that drug-induced AMPK activation in melanoma cells is effective in the induction of a reduction of cell proliferation at least *in vitro*. In particular, upon AICAR- (1 mM for eight days) but not Phenformin-induced AMPK activation, melanoma cells remain viable, still not undergo to apoptotic process and stop proliferating as assessed by MTS/PMS test.

4.2.3 Activated AMPK regulates cell cycle progression through the up-regulation of the cell cycle inhibitor p21.

The observed decrease of proliferation rate as a consequence of AMPK activation in melanoma cells was accompanied by a progressive cell cycle arrest. A panel of melanoma cell lines was tested for cell cycle profile upon 1 mM AICAR and 0.5 mM Phenformin treatment for 8 days. In such conditions, all the tested melanoma cell lines blocked cell cycle progression in either the G₀/G₁ phase or the S phase. Representative cell cycle profiles for such behaviors are shown Figure 4.7 A, upper or lower panel respectively. Among a group of 16 analyzed melanoma cell lines, a subset of 5 samples stopped cycling by reducing the percentage of cells in S phase after 8 days of 1 mM AICAR treatment, from 37.97-54% to 7.67-14.53% (AICAR) or 8.93-15.40% (Phenformin). (Figure 4.7 B, left panel). This was not observed in non treated cells. Similar effects were seen upon Phenformin treatment at 0.5 mM (Figure 4.7 B, left panel), which dose is not associated to apoptosis, as previously observed. The second subset of melanoma samples displayed 11 out of 16 cell lines which accumulated in the S

phase with almost no cells detected in G₂/M phase: from 44.49-48.1% to 78.24-80.91 (AICAR) or 69.86-71.26% (Phenformin) (Figure 4.7 B, right panel).

To better unravel the molecular mechanisms of cell cycle arrest in AMPK-activated melanoma cells, expression levels of some signaling molecules was tested. So far, in agreement with the observed cell cycle arrest, an increase of expression levels of the cell cycle inhibitor protein p21 was observed as a consequence of AICAR treatment in a dose-dependent manner in all melanoma samples (Figure 4.7 C). Upon AICAR treatment, ACC phosphorylation was up-regulated as a consequence of AMPK activation, and an increment of p21 levels was observed after 24 hours treatment with 1 mM and 2 mM AICAR. Figure 4.7 C shows a representative experiment for 4405P melanoma cell line.

These data showed that drug-induced AMPK activation in melanoma cells leads to the block of cell cycle progression. Cell cycle arrest is achieved through the up-regulation of the cell cycle inhibitor p21, which occurs after 24 hours of AMPK activation. Other molecules involved in the presented cell cycle arrest mechanism remain to be identified.

4.2.4 Evaluation of the SA- β -galactosidase activity upon AMPK activation.

As previously shown for the “double mutant” NRAS/BRAF cell model (see Chapter 3.2.2.3), beside cell proliferation arrest presented in the previous paragraph, AMPK activation could be related to cellular senescence in melanoma samples. In order to test this hypothesis, SA- β -galactosidase (β -gal) activity as a marker for cell senescence (Dimri et al., 1995) was measured in different melanoma samples treated or not with AICAR. Measurements of β -galactosidase activity was performed either by testing for

development of blue color after cellular staining with the X-gal substrate, or by measuring fluorescence intensity of the fluorescein di- β -D-galactopyranoside (FDG) substrate by flow-cytometry.

By either X-gal staining or FDG fluorescence intensity, variable basal levels of β -gal activity in a panel of melanoma samples were detected. In Figure 4.8 A, representative images relative to one β -gal negative (Dauv) and one positive (4405P) melanoma cell line displaying respectively lower and higher fluorescence intensity by FACS analysis are shown (Figure 4.8 A, upper panel). Such variability in β -gal basal activity was evident also by X-gal staining (Figure 4.8 A, lower panel). Melanoma cells were then treated with 1 mM Phenformin and the development of blue color or the increase of fluorescence intensity were evaluated after 11 days. No visible differences were notable by cellular X-gal staining. However, by using the more sensitive FDG fluorescent method, discrimination and quantification of the increment of β -gal activity was possible. As shown in Figure 4.8 C, Dauv reached higher fluorescence intensity after 11 days of 1 mM Phenformin treatment.

These results indicate that, in a panel of different melanoma samples showing different basal β -gal activity, increased levels of such senescence marker can be achieved upon AMPK activation by Phenformin treatment for 11 days. Possibly, longer treatment time would allow for the achievement of a more striking effect, which could be detectable also by the standard X-gal cellular staining. Moreover, these data need to be considered preliminary and should be corroborated in a wider panel of melanoma cell lines.

4.2.5 Modulation of adhesion molecules as a response of pAMPK induction upon drug treatment.

As observed in chapter 4.2.2, melanoma cells treated or not with either AICAR or Phenformin showed different cell morphology (data not shown). When treated with Phenformin, since the 3rd day of treatment melanoma cells assumed a more spherical shape in comparison to the not treated cells. *This effect was not detected upon AICAR treatment (data not shown).* After the 3rd day of treatment, Phenformin-treated cells spontaneously detached from the plate surface, while AICAR-treated ones still remain flat and anchored to the plastic.

In order to evaluate the potential effects of AICAR and Phenformin treatment on cell adhesion, expression of such cell surface molecules as integrins and VE-Cadherin was evaluated in melanoma cell lines. As a confirmation of the reported observations, upon 4 days of Phenformin treatment, $\alpha 2\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, $\alpha 4\beta 4$, $\alpha 3$, $\beta 4$, $\alpha 1\beta 1$ and VE-Cadherin decreased their expression (Figure 4.9 A). In contrast, all the tested adhesion molecules were slightly up-regulated or not modulated upon AICAR treatment, in different melanoma cell lines (Figure 4.9 B).

These data suggest that Phenformin and AICAR do not work through the same way on melanoma cells. Indeed, the two drugs have different mechanisms of action in cells. Hence, they might induce different cell effects independently of AMPK activation. Phenformin treatment demonstrated to produce more toxic effects on cells. Therefore, in

order to achieve the expected effects of reduced cell proliferation and cell cycle arrest without undesirable toxic side effects, AICAR treatment is preferable.

4.3 Discussion

Results obtained in this section of the thesis showed that AMPK activity and its phosphorylation levels can be *in vitro* modulated in melanoma cells by using AICAR or Phenformin treatment. However, both basal and drug-induced kinase activities appeared to be lower in melanoma cells than in control non neoplastic cells, thus suggesting that melanoma cells might have an intrinsic mechanism to prevent AMPK activation or to impair drug response in tumor cells. The increment of AMPK phosphorylation causes elevated phosphorylated acetyl-CoA carboxylase (pACC) levels, which are indicative of AMPK activation. When AMPK is activated and phosphorylated, melanoma cells decrease proliferation rate and progressively arrest the cell cycle. The observed block of cell cycle in a panel of melanoma samples was in agreement with previous reports in literature. In more details, a number of studies showed an AICAR-dependent cell cycle arrest in the G₀/G₁ phase (Jones et al., 2005; Xiang et al., 2004), as emerged in one subset of melanoma samples analyzed in the present work, while others reported cell cycle block with cell accumulation in the S-phase (Zakikhani et al., 2006; Rattan et al., 2005), as the case of the second subset of melanoma cell lines. Furthermore, upon AMPK activation, the progressive inhibition of cell proliferation is accompanied by the up-regulation of cell cycle inhibitor p21.

Present data so far collected suggested that Phenformin and AICAR are likely not to work through the same pathway on melanoma cells. Although they both succeed in

activating AMPK protein, they seem to induce different cellular additional effects. Yet Phenformin treatment showed more toxic collateral effects on melanoma cells. In contrast, proliferation of melanoma cells can be inhibited by AICAR treatment without inducing apoptosis or necrosis. For this reason, in order to achieve the expected effects of reduced cell proliferation and cell cycle arrest upon AMPK activation without undesirable toxic side effects, AICAR treatment is preferred to be used.

Given previous observation on the induction of senescence upon AMPK phosphorylation in the NRAS/BRAF “double mutant” melanoma cells (Petti et al., 2006), AMPK activation in melanoma cells could be related to the senescent phenotype. Notably, the quantitative FDG method, which can be used for β -gal activity revealing, indicated that fluorescence intensity increases upon Phenformin treatment in melanoma samples. This effect suggests a progression towards senescence phenotype as a consequence of drug-induced AMPK activation. However, further evidence of such an effect has to be provided.

Recent data from literature showed that AMPK activation results in proliferation inhibition and cell cycle arrest in both cancer and normal cells. After either AICAR treatment or cell infection with a vector expressing constitutive active AMPK, cell cycle of mouse embryonic fibroblasts (MEFs) arrested at the G₁/S transition despite sufficient glucose conditions involving a p53-dependent mechanism (Jones et al., 2005). In agreement, AICAR stimulation inhibits cell growth and increases expression of p21 in human prostate cancer cells (Xiang et al., 2004). Further evidence of the anti-proliferative AMPK-mediated effects of AICAR are provided from Rattan et al., (2005) which show that activated AMPK can block different tumor cell lines in the S-phase of cell cycle by

inducing the expression of p21, p27 and p53 proteins and by inhibiting Akt phosphorylation. Also Metformin, a biguanide as well as Phenformin, commonly used in the treatment of type 2 diabetes mellitus, acts as a growth inhibitor in epithelial cells activating AMPK pathway (Zakikhani et al., 2006). These reports also supported the hypothesis that the mechanism of cell cycle arrest by AMPK activation involves accumulation of the tumor suppressor protein p53 by phosphorylation of its Ser-15 residue, and the accumulated p53 protein up-regulates at least one of the CDKIs, such as p21, by a transcriptional mechanism. Whether phosphorylation of Ser-15 on p53 is mediated by AMPK itself or by another protein kinase, which is co-immunoprecipitated with AMPK, has not been elucidated. Importantly, the aminoacidic sequence around Ser-15 does not fit best with the AMPK consensus recognition motif. In agreement, in our study the induction of p21 levels after AICAR stimulation is likely to be p53-independent, since no increase of Ser15-p53 phosphorylation was observed in some treated melanoma cell lines (data not shown). Future studies are needed to provide the complete understanding for the regulation of p53 protein by AMPK activation.

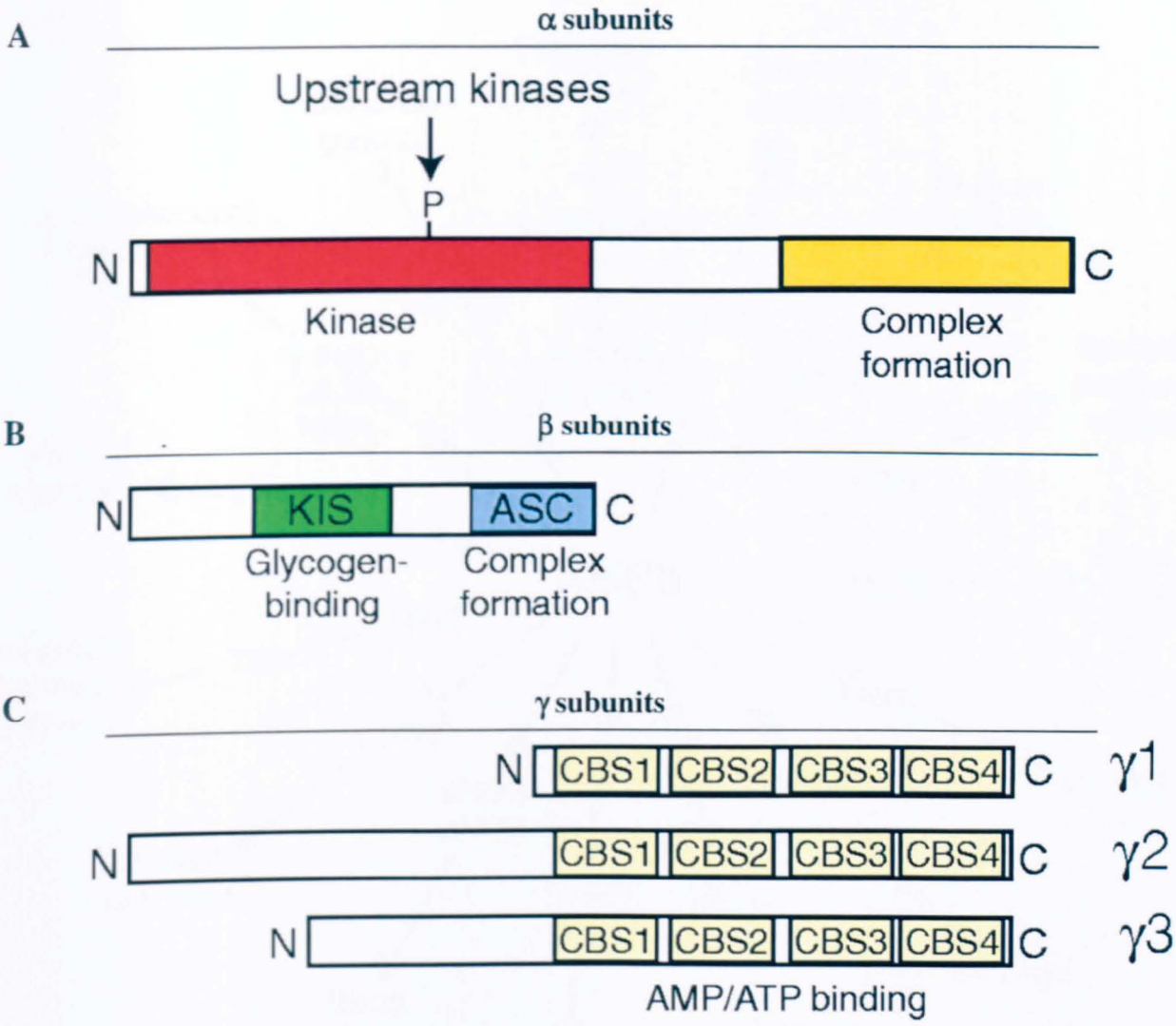
Some evidences also show that LKB1 associates with p53 (Karuman et al., 2001) and induces p21 up-regulation resulting in cell cycle arrest in a p53-dependent manner in G361 melanoma cells (Tiainen et al., 2002). Overexpression of wild type LKB1 in two cancer cell lines, HeLa and G361, which do not express endogenous LKB1, suppressed the proliferation of these cells by inducing a G₁ cell cycle arrest (Tiainen et al., 1999). Catalytically inactive LKB1 mutants fail to suppress cell growth, indicating the proliferation-inhibitory and anti-tumor effects of LKB1. This failure of LKB1 activity impairs its downstream signalling on AMPK (Forcet et al., 2005), but so far no published

work has shown the direct evidence for LKB1-AMPK association in AMPK-dependent cell cycle arrest. However, all melanoma cell lines analyzed in the present study expressed LKB1 at protein level (data not shown), thus ruling out the possibility that the effects on cell proliferation upon AMPK activation can be impaired.

An additional pathway potentially implicated in the cell growth inhibition dependent on AMPK activation is the PI3K-Akt pathway. Constitutive activation of this signaling pathway has been reported in many cancers including glioblastoma, melanoma, and advanced prostate cancer. AMPK inhibits mTOR signaling downstream of Akt, and inhibition of mTOR pathway has been reported to inhibit tumor growth and metastasis in experimental animal models as well as in cultured cells. In the next future, the involvement of such a pathway, which might have relevant importance in the control of cell growth, will be deeply investigated in melanoma cells.

As discussed above, AMPK can regulate a variety of signaling pathways. Therefore, it will be interesting to determine which pathways regulated by AMPK are most involved in the inhibition of cancer cell proliferation. To this aim, further studies are required for a full comprehension of this issue. Deeper investigations about the molecular targets of activated AMPK are needed in order to elucidate the mechanism leading to the cell cycle arrest. Nevertheless, given the central role of AMPK in the control of cell proliferation and cell cycle, as supported also by our latest results, AMPK activation could represent a possible new target for the development of novel potential therapeutic strategy for cancer treatment.

FIGURES OF CHAPTER 4



(From Hardie DG, *J Cell Sci* 2004)

Figure 4.1. Conserved domains in AMPK subunits: α subunits, β subunits and γ subunits.
The proposed function of each domain is indicated. **A.** α subunits. **B.** β subunits. **C.** γ subunits. The two isoforms of the α ($\alpha 1$, $\alpha 2$) and β ($\beta 1$, $\beta 2$) subunits have very similar structures, but the three isoforms of the γ subunit ($\gamma 1$, $\gamma 2$, $\gamma 3$) contain variable N-terminal regions of unknown function, and are drawn separately.

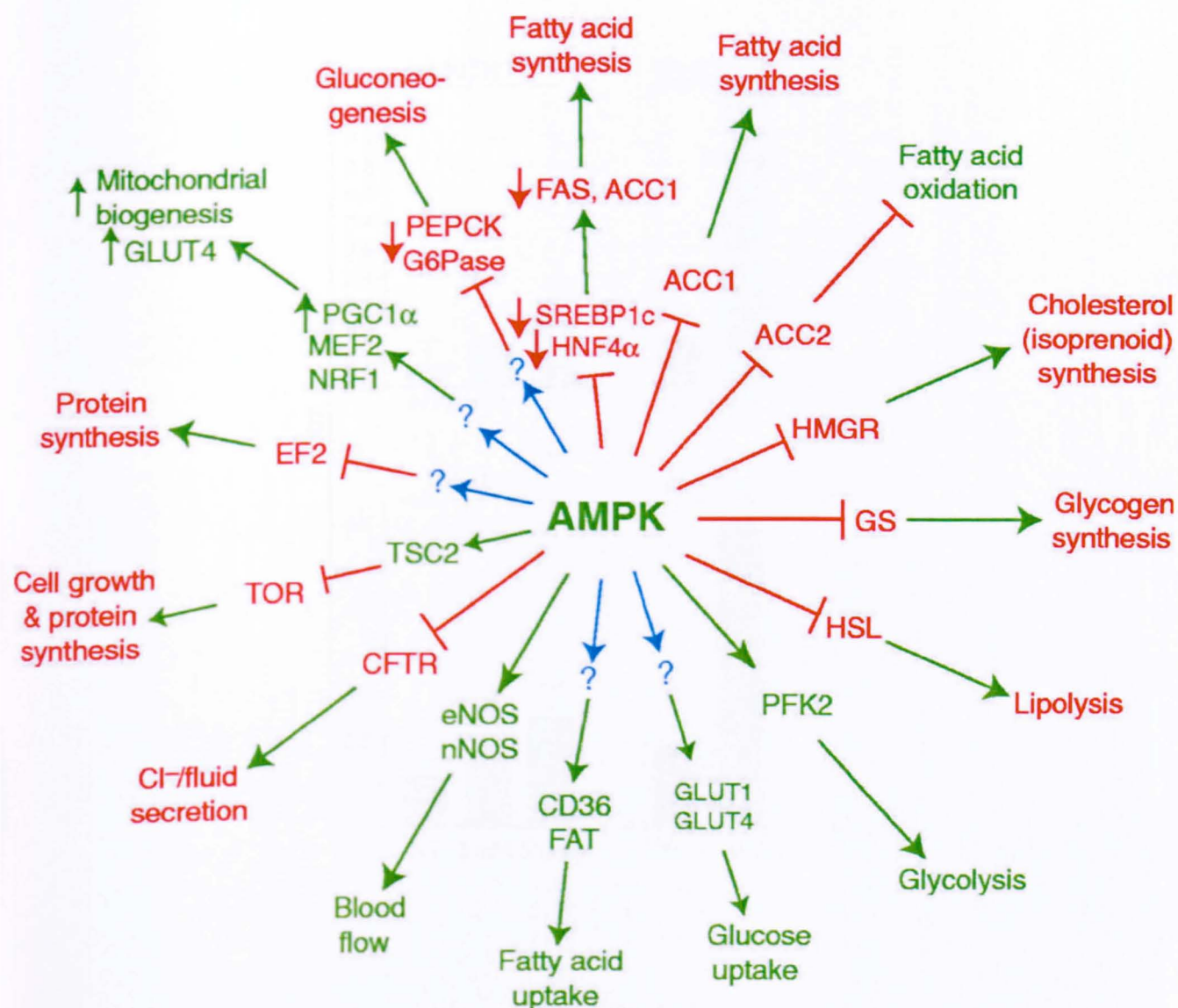


Figure 4.2. Targets for AMPK.

Target proteins and processes activated by AMPK activation are shown in green, and those inhibited by AMPK activation are shown in red. Where the effect is caused by a change in gene expression, an upward-pointing green arrow next to the protein indicates an increase, whereas a downward-pointing red arrow indicates a decrease in expression. Abbreviations: ACC1/ACC2, 1 (α) and 2 (β) isoforms of acetyl-CoA carboxylase; CD36/FAT, CD36/fatty acid translocase; CFTR, cystic fibrosis transmembrane regulator; EF2, elongation factor-2; eNOS/nNOS, endothelial/neuronal isoforms of nitric oxide synthase; FAS, fatty acid synthase; G6Pase, glucose-6-phosphatase; GLUT1/4, glucose transporters; GS, glycogen synthase; HMGR, 3-hydroxy-3-methyl-CoA reductase; HSL, hormone-sensitive lipase; MEF2, myocyte-specific enhancer factor-2; NRF1, nuclear respiratory factor-1; PEPCK, phosphoenolpyruvate carboxykinase; PGC1, peroxisome proliferator-activated receptor-co-activator-1; TOR, mammalian target of rapamycin.

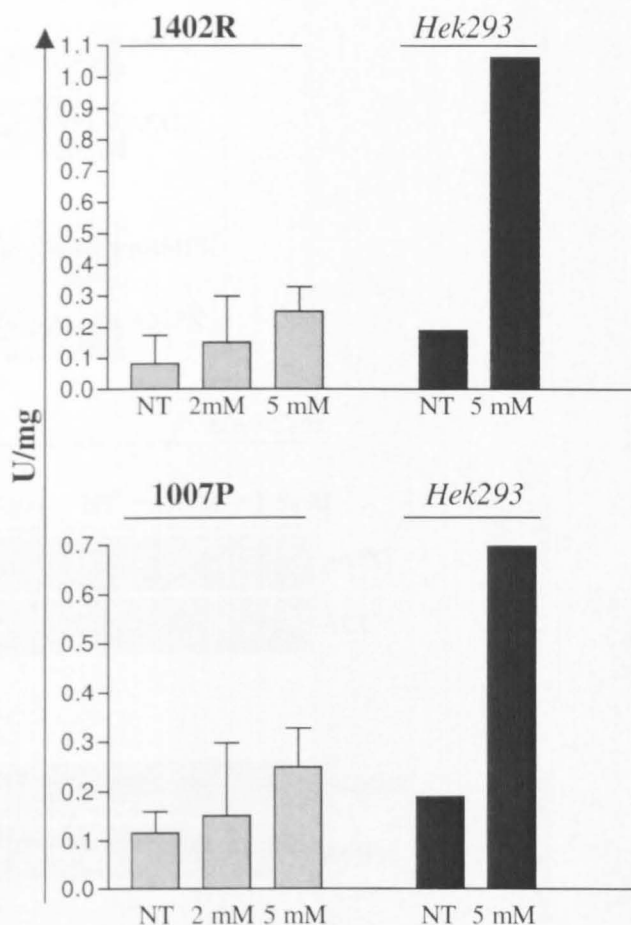


Figure 4.3. Effects of Phenformin on AMPK activity.

AMPK activity was measured by the IP kinase assay (see Chapter 2.15, in Materials and Methods) using AMARA peptide. Hek293 treated or not with 5 mM Phenformin, were used as positive control for the AMPK activation upon Phenformin treatment. NT: not treated cells; 2 mM, 5 mM: doses of Phenformin. U/mg: units of enzyme activity/mg of protein.

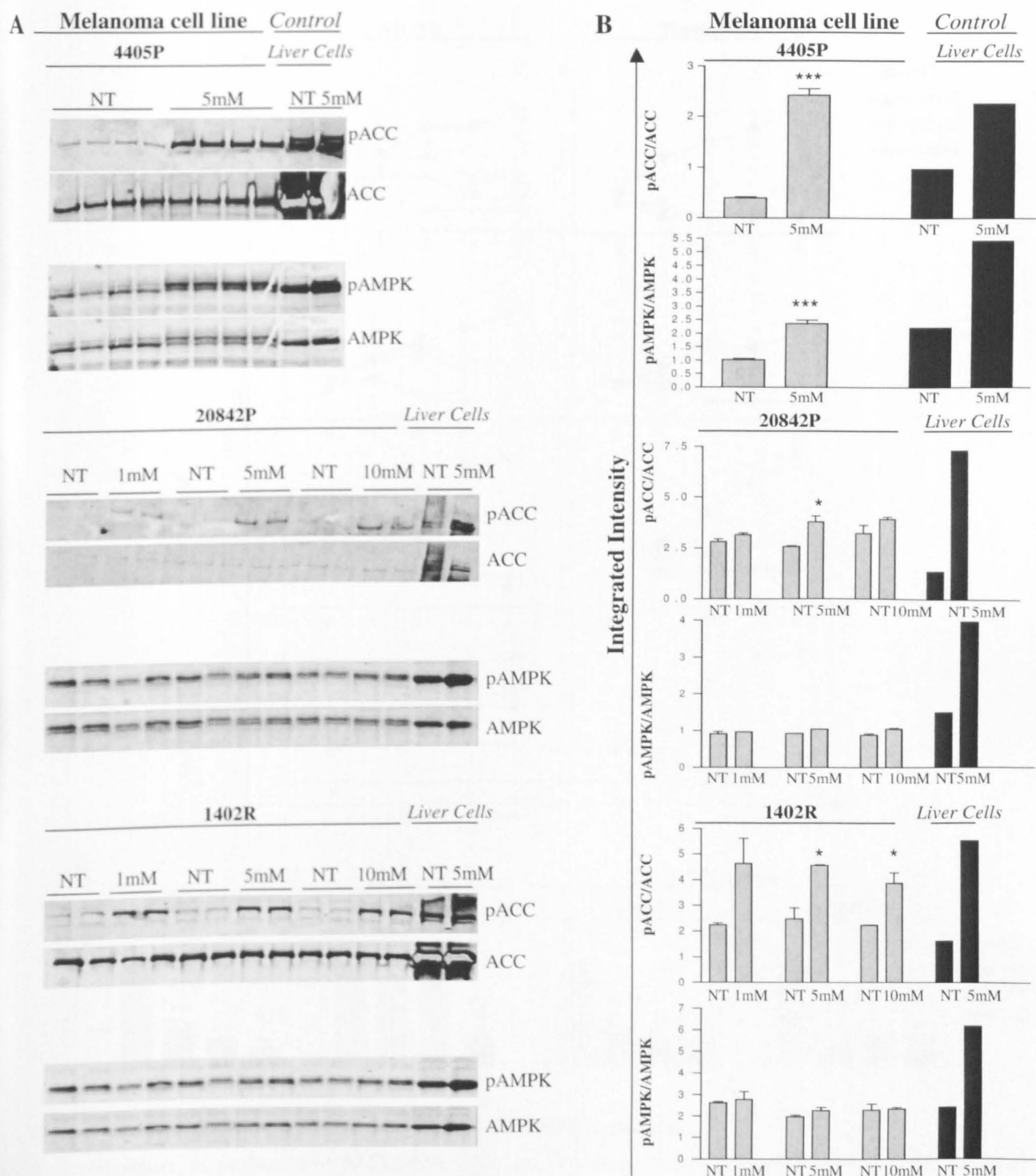


Figure 4.4. Effects of AICAR on Ser79-ACC (pACC) and Thr172-AMPK (pAMPK) expression in melanoma cells.

A. Western Blot images of both pACC and pAMPK expression upon AICAR treatment. **B.** Quantification of pAMPK and pACC expression modulation by infra-red Li-Cor Odyssey imaging, expressed as the ratio between band intensity of the phosphorylated and of the total protein.***: $p < 0.001$ and *: $0.05 < p < 0.01$, compared with NT cells. In each experiment, lysates from human liver cells treated or not with 5 mM Aicar for 2 hours were used as a control for increased pACC and pAMPK expression. NT: Not Treated; 1mM, 5mM, 10mM: doses of AICAR; each treatment was performed for 2 hours.

A

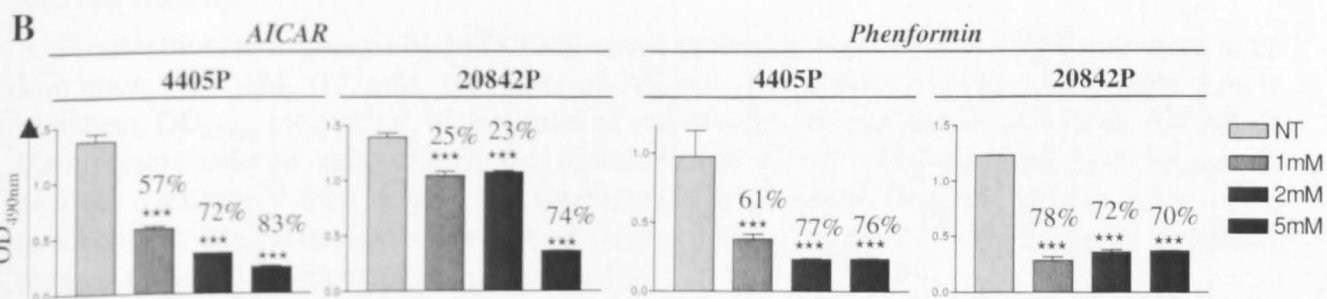
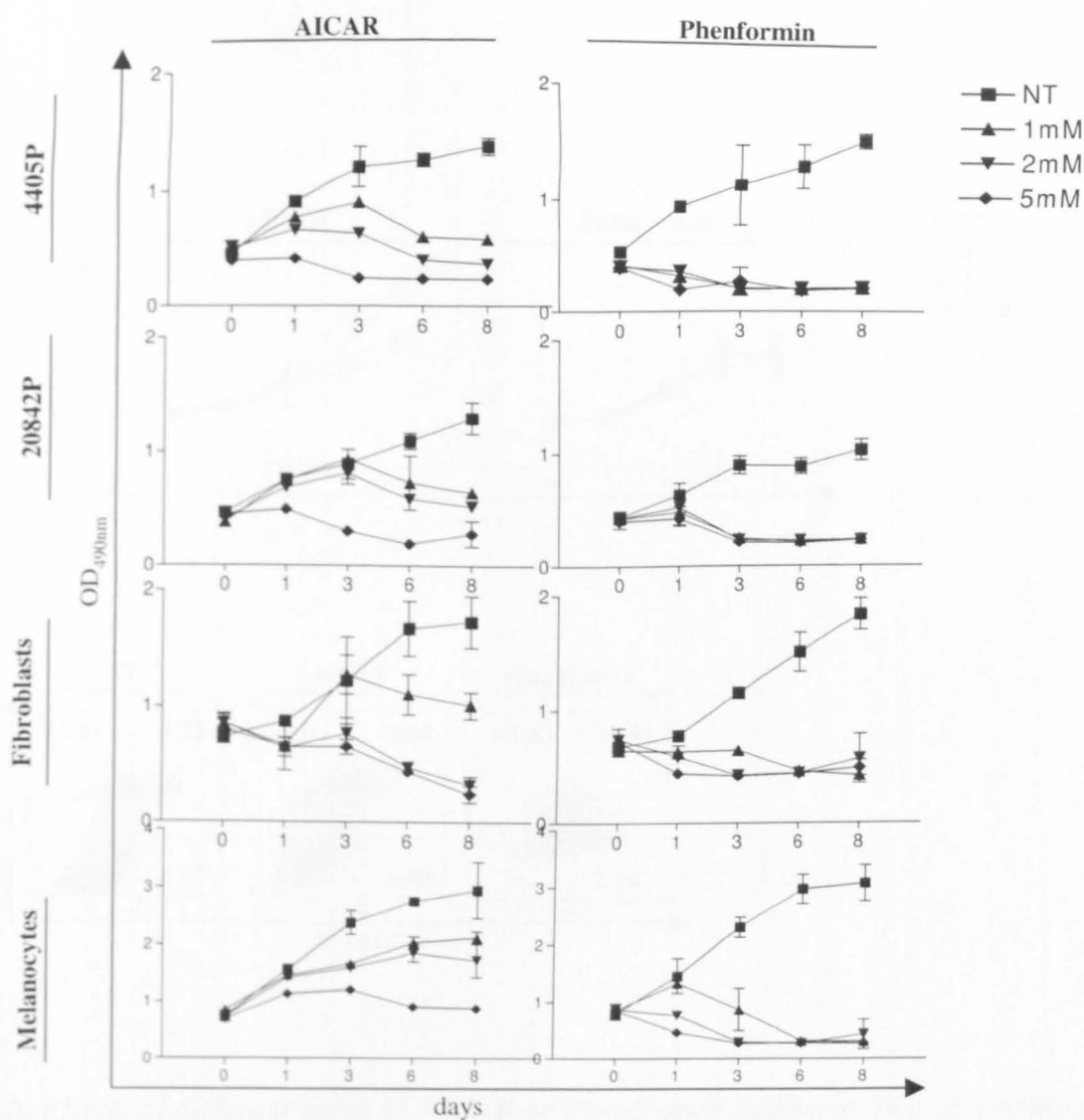


Figure 4.5. Proliferation of melanoma cells upon AMPK activation.

A. Proliferation, as evaluated by MTS/PMS assay, of two melanoma cell lines (4405P, 20842P), normal fibroblasts and normal melanocytes, upon AMPK activation after AICAR (left panel) or Phenformin (right panel) treatment. OD_{490nm} mean \pm s.d. of triplicates of one of three experiments are shown. **B.** OD_{490nm} values of AICAR treated cells at day 8 were significantly different at all different doses; ***: $p < 0.001$ compared with control Not Treated cells. NT: Not Treated.

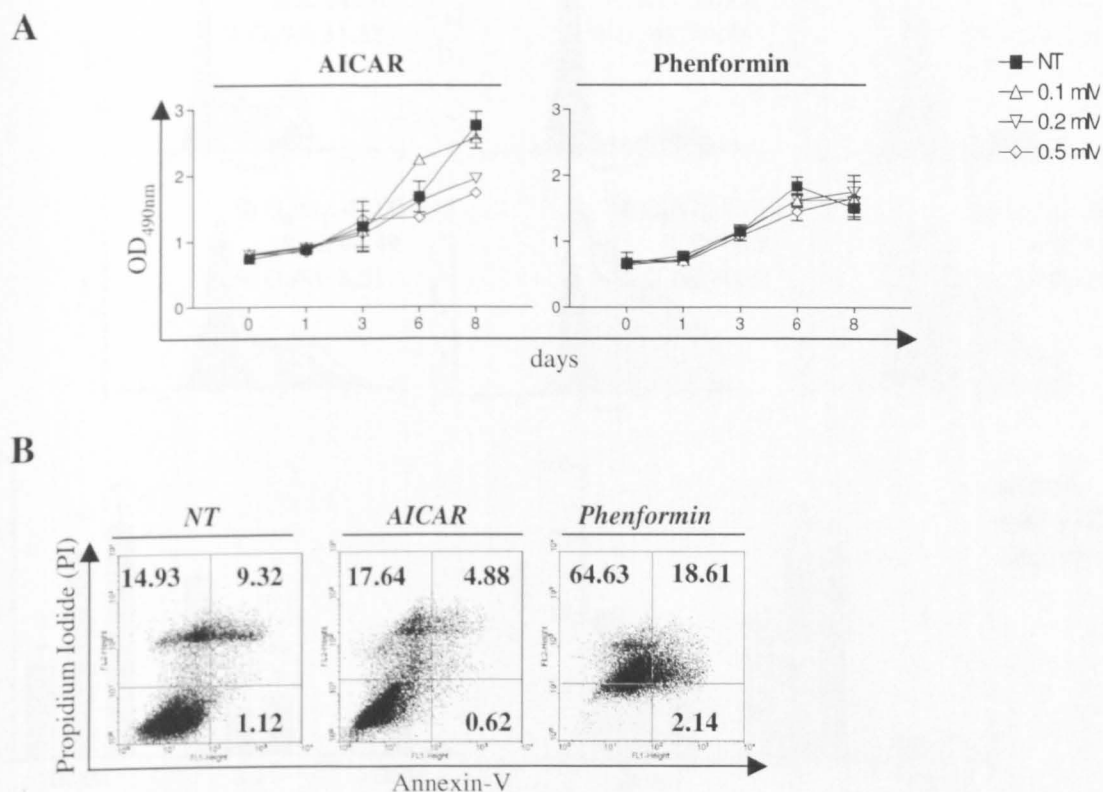


Figure 4.6. Effects of different doses of AICAR or Phenformin treatment on cell proliferation and cell viability.

A. Proliferation, as evaluated by MTS/PMS assay, of fibroblast cells upon AMPK activation after low doses (0.1 mM, 0.2 mM, 0.5 mM) of AICAR (left panel) or Phenformin (right panel) treatment. OD_{490nm} mean \pm s.d. of triplicates of one of three experiments are shown. **B.** AICAR or Phenformin-induced apoptosis in the representative 4405P melanoma cell line. Extent of necrosis (annexin-V-/PI+, upper left quadrant) and of early (annexin-V+/PI-, lower right quadrant) or late (annexin-V+/PI+, upper right quadrant) apoptosis induced by the indicated drugs is indicated as percentage of positive cells.

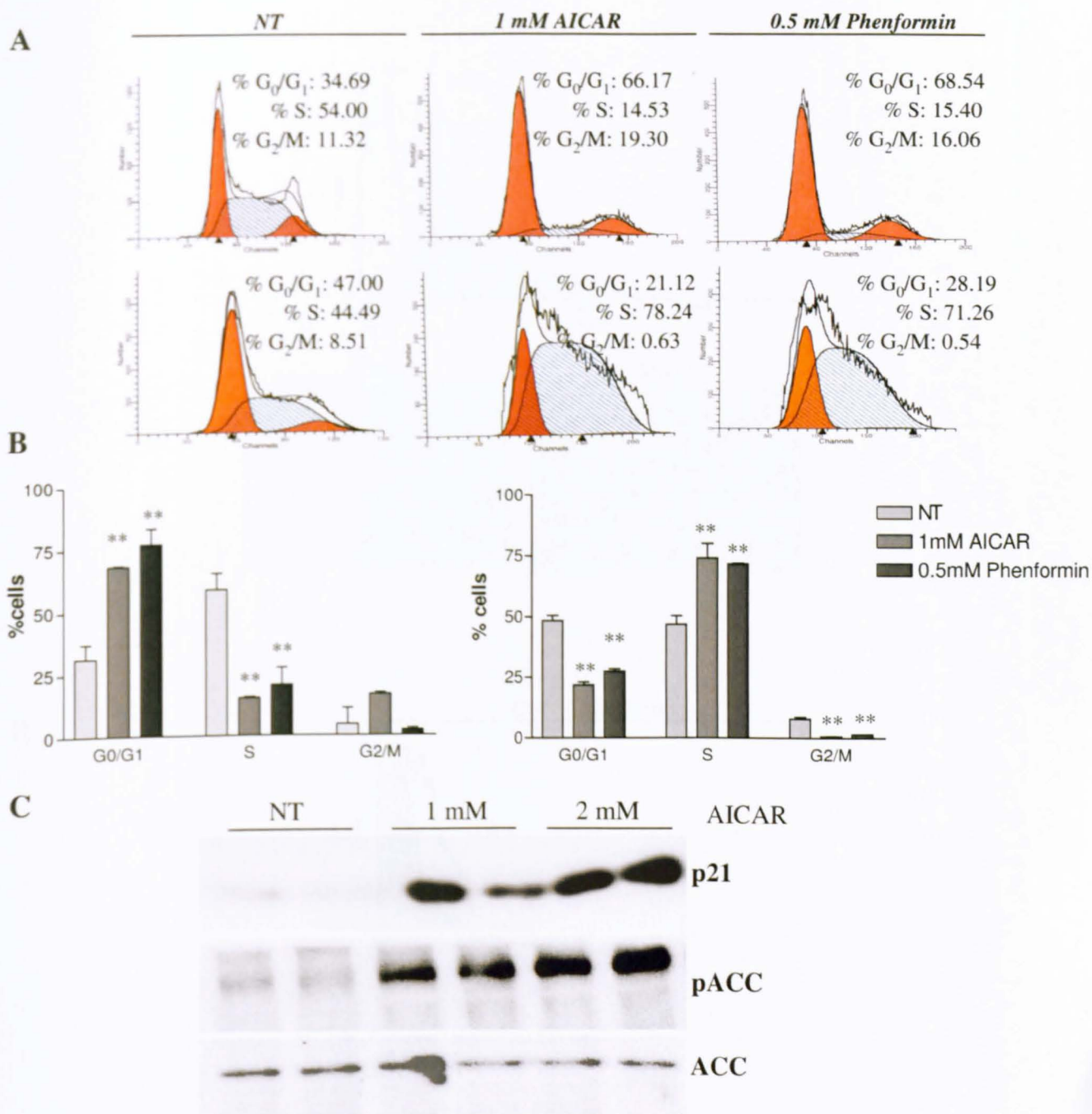
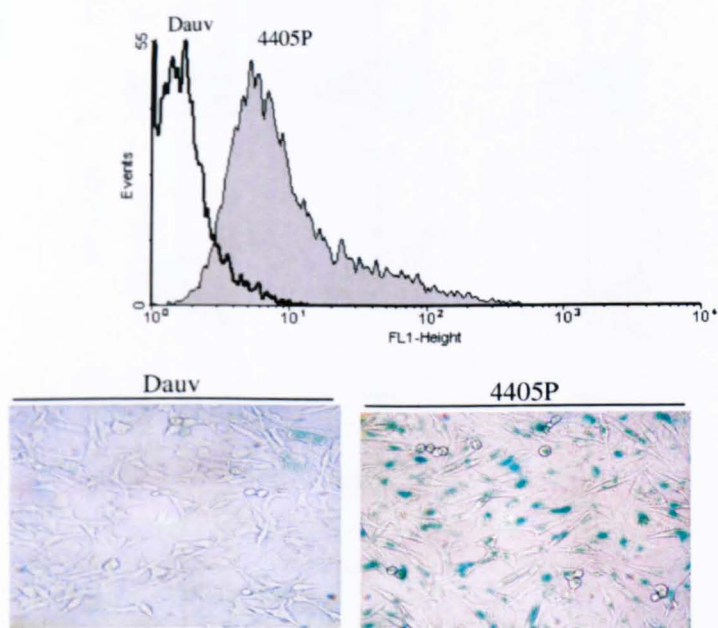


Figure 4.7. Effects of AMPK activation on cell cycle in melanoma cells.

A. Cell cycle profile of melanoma cells treated or not with 1mM AICAR or 0.5 mM Phenformin; *upper panel*: cell cycle arrest in G₀/G₁ phase, as represented for 4405P melanoma cell line; *lower panel*: cell cycle arrest in S phase, as represented for 1402P melanoma cell line. **B.** Cell cycle analyses of 4405P (left panel) and 1402P (right panel) relative to three independent experiments represented as percentage of cells in G₀/G₁-, S-, G₂/M- phases. NT: Not Treated cells. **: 0.05 > *p* > 0.001, compared with NT. **C.** Western blot analysis of p21, pACC and total ACC in melanoma sample after 1 mM or 2 mM AICAR treatment for 24 hours. NT: Not Treated cells; 1 mM, 2 mM: doses of AICAR.

A



B

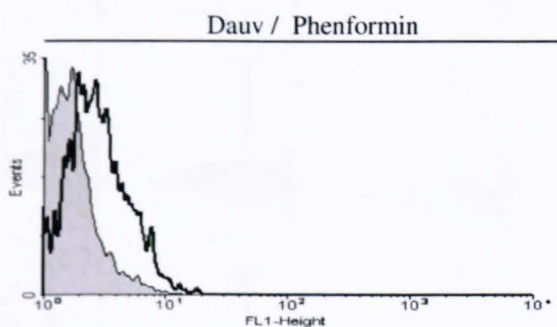
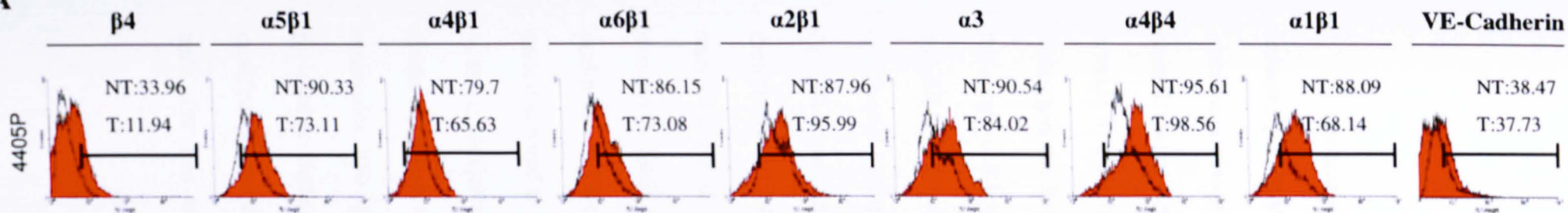


Figure 4.8. SA-β-galactosidase (β-gal) activity in melanoma cell lines.

A. Upper panel: Fluorescence intensity relative to FDG substrate of β-gal in two melanoma samples; gray hystogram: Dauv, empty hystogram: 4405P. Lower panel: Basal SA-β-galactosidase activity as assessed by X-gal cellular staining: no β-gal activity detected in Dauv and higher activity in 4405P. Fluorescence intensity is represented on the abscissa and the relative number of cells is represented on the ordinate. **B.** FACS analysis of the fluorescein signal produced by β-galactosidase hydrolysis of FDG in one melanoma sample (Dauv) treated (empty hystograms) or not (grey hystograms) with Phenformin at 1 mM of concentration for 11 days.

A



B

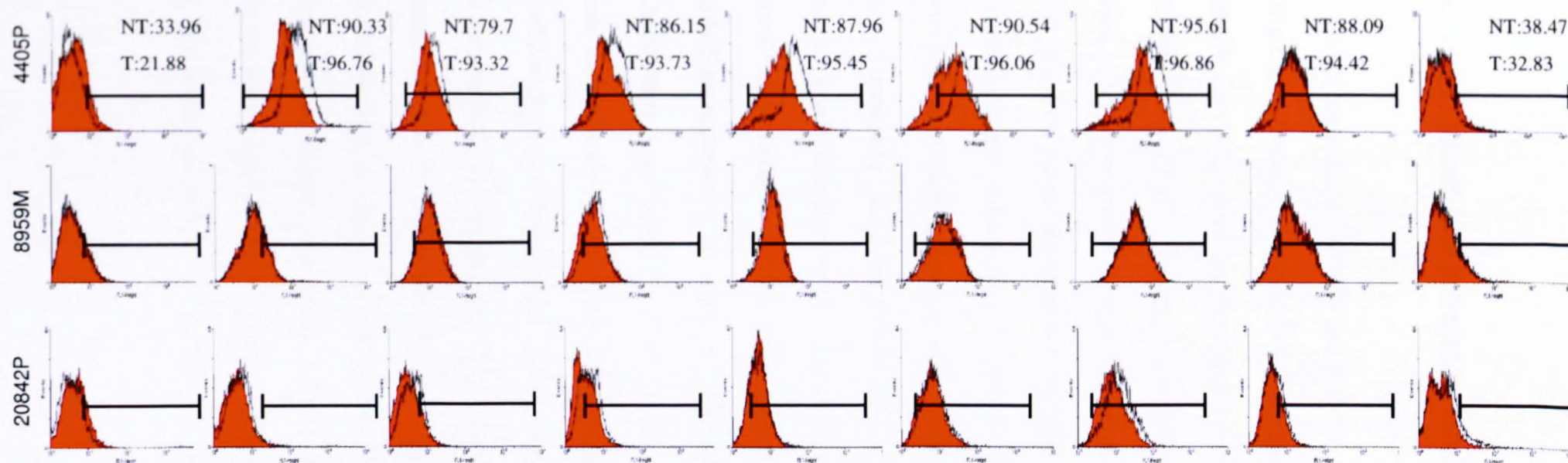


Figure 4.9. Modulation of adhesion molecules upon drug treatment.

A. Flow cytometry analysis for cell-surface molecules ($\alpha v\beta 3$ integrin, $\beta 1$, $\beta 4$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$ integrin subunits, VE-Cadherin) in 4405P cells treated (T, empty histograms) or not (NT, red histograms) with 1 mM Phenformin for 4 days. B. Flow cytometry analysis for cell-surface molecules ($\alpha v\beta 3$ integrin, $\beta 1$, $\beta 4$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$ integrin subunits, VE-Cadherin) in 4405P, 8959M and 20842P cells treated (empty histograms) or not (red histograms) with 1 mM AICAR for 4 days. NT: Not Treated; T: Treated. — : marker was set based on the histogram relative to the staining with FITC-labeled secondary antibody only.

6. CONCLUSIONS AND FUTURE PLANS

The aim of the present study was to identify new possible molecular targets for inducing proliferation arrest in melanoma cells. In spite of their high frequency in human melanoma, oncogenic *BRAF* and *NRAS* mutations are generally mutually exclusive. The mutual exclusivity of *NRAS*^{Q61R} and *BRAF*^{V600E} suggests that their coexistence may not provide a selective advantage for tumor growth, or may even be selected against during tumorigenesis. In the first part of the project the mutual exclusion of the two mutations at the single cell level was demonstrated. Subsequently, in order to verify whether *NRAS* and *BRAF* activating mutations were synthetic lethal, a model system with constitutive or conditional co-expression of oncogenic *NRAS* and *BRAF* in the same cell was created. Growth arrest and senescence occurred in such double mutant cells. Further research was then focused on the identification of genes which are significantly modulated when both oncogenic *NRAS* and *BRAF* are expressed in the same cell, compared to cells with single mutations. Such modulated genes could represent relevant targets for suppressing cell proliferation. To this aim, molecular profiling by MicroArray analysis of double mutant cells compared to cells with a single mutated oncogene was performed. Such analysis led to the identification of candidate genes implicated in cell proliferation, cell cycle, cell death, response to stress, response to wound and immune response. Among these molecules, two specific targets, such as Lipocalin2 (LCN2) and LOXL3, were selected for further studies. Such molecules might play a relevant role in the control of cell proliferation not only in the double mutant cell model, but also in general in melanoma cells. For this reason, the impact of LCN2 and LOXL3 expression on tumor cell

phenotype need to be assessed in a wide panel of melanoma samples. Preliminary results showed that LCN2 can be silenced in melanoma cells expressing high basal levels of the protein. Biological consequences of the induced silencing have to be evaluated in melanoma cells. To this aim, in the next future a number of melanoma cell lines constitutively expressing high levels of LCN2 will be selected and transfected with specific siRNA in order to silence its expression. On the other hand, a set of melanoma cell lines identified as low expressors of LCN2 will be transfected with specific constructs for the expression of LCN2. Low and high LCN2 induced-expressor melanoma cells will be tested for different cell features such as cell proliferation, cell cycle, senescence. With this strategy it might be possible to clarify the role of LCN2 protein in influencing tumor cell behavior. An analogous approach should be used for LOXL3 as well. In the optic of the investigation about proliferative impairment in melanoma cells, all the amount of data obtained from the microarray analysis provides a rich source of potential candidate genes which can mediate cell behavior characteristics as important as cell proliferation and cell cycle progression. Therefore, from the obtained data sets, other possible molecules which can reveal a tumor suppressive function can be extrapolated. Hence, further investigations may be focused on additional genes from this list, in order to enlarge the panel of potential mediators of cell proliferation arrest. The identification of new mediators of proliferation arrest in melanoma cells may open the way to the discovery of novel strategies for innovative therapeutic approaches in melanoma treatment.

As presented in the second part of the thesis, AMPK was previously identified as a new possible mediator of the senescence/growth-arrest mechanism observed in the double

mutant model. Therefore, AMPK might play a relevant role in melanoma cells as concerning to cell proliferation control. So far, collected data showed that AMPK activation can lead to growth arrest in melanoma cells. Furthermore, AMPK activation in melanoma cells could be related to the senescent phenotype. For these reasons, in order to identify a possible mediator of proliferation arrest in human melanoma, the role of AMPK molecule was further investigated in a panel of human melanoma cell lines. AMPK expression and activation upon drug treatment in melanoma cells was evaluated and its role was tested as tumor suppressor. In addition to cell proliferation and cell cycle arrest evaluated by standard methods, the quantitative FDG assay, which can be used for β -gal activity revealing, indicated that upon AICAR or Phenformin treatment in melanoma samples there is a progression towards senescence phenotype. However, further evidence of such an effect has to be provided, testing the same effect on various melanoma cell lines. As discussed above, AMPK can regulate a variety of signaling pathways. Therefore, it will be interesting to determine which pathways regulated by AMPK are most involved in the inhibition of cancer cell proliferation. To this aim, in order to elucidate the mechanism leading to the cell cycle arrest, a macroarray study focused on cell cycle related molecules will be performed in the very next future. In addition, *in vivo* consequences of the activation of AMPK upon AICAR treatment in mice need to be evaluated. Previous evidences suggest that injected tumors in mice treated with AICAR should stop growing or at least should modulate the expression of typical markers of proliferation such as PCNA, Ki67 and, on the opposite way, β -gal. As a future implication of this study, given the central role of AMPK in the control of cell proliferation and cell cycle, which evidence is supported also by our latest results, AMPK

activation could represent a possible new target for the development of novel potential therapeutic strategy for cancer treatment.

In conclusion, starting from a double mutant cellular model for inducing melanoma cells growth arrest, candidate genes linked to the NRAS/BRAF mutations were identified as potentially involved in cell cycle and proliferation arrest and senescence in melanoma cells. Interesting targets, such as LCN2, LOXL3 and additional genes which can be extrapolated from all these amount of data, may be tested for their role in proliferation inhibition in melanoma cells. In addition, preliminary evidences already exist that AMPK might be a valid new possible target for drug therapy of human melanoma.

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